STUDIES ON NICOTINE *N*-DEMETHYLATION IN CELL SUSPENSION CULTURES OF *NICOTIANA TABACUM* L CV. WISCONSIN-38

by

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A Thesis Presented in Fulfilment of the Requirements
for the Degree of Doctor of Philosophy

University of Edinburgh

1994

Declaration

I hereby declare that this thesis was composed by myself, and the work described herein to be my own, except where indicated otherwise.

Acknowledgements

I would like to express my gratitude to my supervisor, Professor M. M. Yeoman, for his invaluable advice and guidance throughout the experimental work and, in particular, for his every efforts in the preparation of this thesis. Also, I would like to thank Dr. M. B. W. Miedzybrodzka for her general help with my experimental work, Dr. S. Fry for his valuable discussion on the enzymology work, Dr. P. Smith for his support, Miss. Z. Gowler for her kindness and guidance concerning my English language; Mr. J. Anthony and Mr. J. Findlay for their help in photography and TEM respectively, and Mrs. E. Raeburn and Mr. J. Durrie for their secretarial assistence throughout my study. In addition, thanks are due to Mr. R. Zarate and Miss. H. Li with whom I have gone through my Ph.D. study and experienced friendship and understanding. Moreover, my thanks are given to Dr. W-P. Lu and his wife, Y-C. Xu, for being there and all their kindness over the last four years.

I would like to acknowledge Rothmans International Services Ltd. for their financial support over the last three years and for carrying out GC-MS analysis.

However, most of all, I wish to express just how much I am indebted to my parents, Professor S. Hao and Professor M-Y. He, to whom this thesis is dedicated, for their love, education, encouragement and understanding since my birth. Also, I am grateful to my brother, Mr. D-S. Hao, and his wife, Mrs. C-Y. Zhao, for their support and looking after my parents during my absence, and to the memory of my grandmothers, who passed away during my Ph.D. study, for every childhood happiness they gave to me.

Last but not least, I would like to express my appreciation to my fiancée, Lin, to whom this thesis is also dedicated, for her sincere love, patience and understanding which enabled me to complete this thesis.

Dong-yun Hao
1994

Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
BDMA	benzyldimethylamine
°C	temperature in degree centigrade
ca.	approximately
CHAPS	3'-[(3'-cholamidopropyl)dimethylaminio]-1'-propanesulfonate
$CHCl_3$	chloroform
Ci	unit of radioactivity, curie(s)
CO	carbon monoxide
cpm	count per minute
DDSA	dodecenyl succinic anhydride
DMSO	dimethylsulfoxide
dpm	disintegration per minute
DTT	dithiothreitol
e.g.	for example
EDTA	ethylene diaminetetraacetic acid
et al.	*et alia*
etc.	*et cetera*
Fig(s).	figure(s)
g.	gram(s)
g.	gravitational force
GC-MS	gas chromatography-mass spectrometry
HCHO	formaldehyde
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour(s)
i.e.	that is
Kat	katal, SI unit of enzyme activity
K_m	Michaelis constant
l	litre(s)
M	molar(s)
m.	metre(s)

m (as a prefix)	milli (10^{-3})
min	minute(s)
μ (as a prefix)	micro (10^{-6})
mol	mole(s)
MNA	methylnadic anhydride
MW	molecular weight
N	normal
n	nano (10^{-9})
NAA	naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
ρ (as a prefix)	pico (10^{-12})
PMSF	phenylmethylsulfonyl fluoride
p.s.i.	pound(s) per square inch
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
Rf	retention factor
RITA	radioactivity intelligent thin layer analyser
rpm	revolutions per minute
sec.	second(s)
TEM	transmission electron microscope
TLC	thin layer chromatography
Tris-HCl	*N*-tris (hydroxymethyl) aminomethane-HCl
v/v	volume per volume
via	through
V_{max}	maximal reaction velocity
w/v	weight per volume
UV	ultraviolet

Contents

Abstract

The aim of this project was to investigate the mechanism of bioconversion of added nicotine to nornicotine in cell suspension cultures of *Nicotiana tabacum* L cv. Wisconsin-38 and, in particular, to study the enzymology of this *N*-demethylation.

Preliminary experiments showed that this cell line, which was established and optimised in this laboratory several years before, was still capable of converting added nicotine to nornicotine at high efficiency and was, therefore, suitable for further studies on this reaction.

Radioactive feeding experiments, in which *DL*-[pyrrolidine-2'-^{14}C]-nicotine was added to 10 day old cultures, confirmed that the kinetic pattern of this *N*-demethylation was similar to that of non-radioactive nicotine, and that nornicotine was the major product and was produced intracellularly with a maximum percentage conversion of approximately 70%. The appearance of nornicotine paralleled the disappearance of the added nicotine, although small amounts of four other radioactive-metabolites were observed. One of these metabolites was tentatively identified by GC-MS as *N*-formyl-3'-nornicotine.

The properties of (-)-nornicotine produced from (-)-nicotine by 10 day old cell cultures were determined using both, polarimetry and chiral gas chromatography. The results obtained, which were convincingly consistent, showed that the nornicotine produced was exclusively one enantiomer. This provided strong evidence that the bioconversion of nicotine by tobacco cultures does not involve opening of the pyrrolidine ring. It is possible that the mechanism of nicotine bioconversion by cultured cells might differ from that proposed for the plant, in which a partially racemized mixture of (+)- and (-)-nornicotine has been reported. This could be the result of the opening and closing of the pyrrolidine ring during bioconversion.

A radioactive enzyme assay procedure developed, using cell-free preparations of tobacco cell cultures, has showed for the first time that the enzyme(s) which catalyses this *N*-demethylation is present in tobacco. The enzyme(s), which has been fully characterised, has a K_m of 7.4μM and a V_{max} of 7.6 × 10^{-2}ρKat and appears to be NADPH dependent. Subcellular fractionation of the homogenate using isopycnic

differential centrifugation, together with TEM, showed that most of the enzyme activity was present in the intermediate pellet whilst the maximum specific activity appeared to be associated with the microsomal fraction. Also, the addition of selected possible methyl group acceptors, i.e. putrescine, glycine and ethanolamine, to either dialysed or undialysed enzyme preparations, did not promote enzyme activity, suggesting that the *N*-demethylation is unlikely to be a transmethylation but satisfies some of the primary criteria for cytochrome P-450 involvement. Studies involving Sephadex G-25 gel fractionation showed that molecules smaller than 5,000MW are not involved in this nicotine *N*-demethylation. Finally, the possible enzymatic mechanism involved in this *N*-demethylation is discussed.

Chapter One:

INTRODUCTION

1.1. Tobacco, the Historical Aspects

Tobacco belongs to the genus *Nicotiana*, a member of the family Solanaceae. When Columbus landed in the New World on 11 October 1492, he was offered dried tobacco leaves at the House of the Arawaks. Although the Chinese claimed that they grew and used tobacco long before the discovery of America, no convincing documentation is available (WHO, International Agency for Research on Cancer, 1986). The first tobacco plant was probably introduced to Europe by Jean Nicot (hence the name '*Nicotiana*'), the French ambassador to Lisbon in 1558 (Usher, 1974). However, the word 'tobacco' had become established in North America and survived for common usage (see Encyclopaedia Britannia, Inc., 1966).

The involvement of tobacco in human society probably lies in its medicinal purposes for which smoking was recommended before the 18th century. The smoking habit was first introduced to the UK by Sir Walter Raleigh in 1585 and, into India and China by the Portuguese. In the same year, the first commercial plantings were made in Virginia by John Rolfe (Usher, 1974). Originally, tobacco was smoked in pipes and, the cigarette became gradually widely accepted in the 18th century. Since the first cigarette-machine factories were set up in Havana (Cuba) in 1853, in London in 1856 and in the American colonies in 1860 (see Encyclopaedia Britannia, Inc., 1966), tobacco has played an important role in farm and international trade. At the beginning of 1970s, the annual world tobacco production was about 8,660 million pounds (Usher, 1974).

In contrast to its economic position in the world trade, the positive benefits of tobacco began to fade at the end of the 19th century, when the idea that smoking might cause cancer was clearly expressed (WHO, International Agency for Research

on Cancer, 1986). Thereafter, the association of tobacco smoking with human health has attracted a great deal of attention and has been investigated in many different ways, in particular over the last few decades. It has been reported that tobacco smoking is causally associated with cancer of the lung, larynx, oral cavity and oesophagus, also that smoking is correlated with cancer of the pancreas, kidney and urinary bladder and possibly with cancer of the cervix (Royal College of Physicians, London, 1983). This carcinogenicity of tobacco smoking is apparently related to the constituents of tobacco (Winn, et al, 1981), for which nicotine and other tobacco alkaloids may be responsible.

1.2. Tobacco Alkaloids and Their Biosynthesis

A major reason that people use tobacco as a stimulant is the presence of alkaloids (WHO, International Agency for Research on Cancer, 1986). This is not only because most of the alkaloids are physiological active compounds, but also alkaloids, amino acids, proteins and saccharides in tobacco leaves contribute significantly to the flavour of tobacco smoke (Brunnemann & Hoffmann, 1982). At least 12 different alkaloids have so far been identified in the genus *Nicotiana* (Marion, 1960), but nicotine, nornicotine, anabasine and anatabine are considered to be the main alkaloids (Tiburcio & Galston, 1987). The level and distribution of these alkaloids vary with the species and within the plant (Saitoh, et al, 1985).

According to current knowledge (see Bush, 1981; Leete, 1985 and Friesen, et al, 1992), a scheme of the biosynthesis of the main alkaloids in tobacco plants is summarised in Fig.1.1. The pyridine ring of nicotine, nornicotine, anabasine and anatabine is formed from nicotinic acid of which, quinolinic acid is an immediate

precursor. The dihydronicotinic acid is subject to concerted decarboxylation and reaction with the *N*-methyl-Δ'-pyrrolinium salt (pyrrolidine ring moiety) leading to the formation of nicotine. Further elimination of the methyl group of the pyrrolidine ring from the nicotine molecule produces nornicotine. Anatabine is synthesised through the condensation of two pyridine rings, which are derived from nicotinic acid as with nicotine, whilst anabasine is formed by the condensation of nicotinic acid and the piperidine ring which is derived from lysine. In contrast to the biosynthesis of nicotine, which is relatively well understood, the pathway of formation of the other three alkaloids is at present hypothetical.

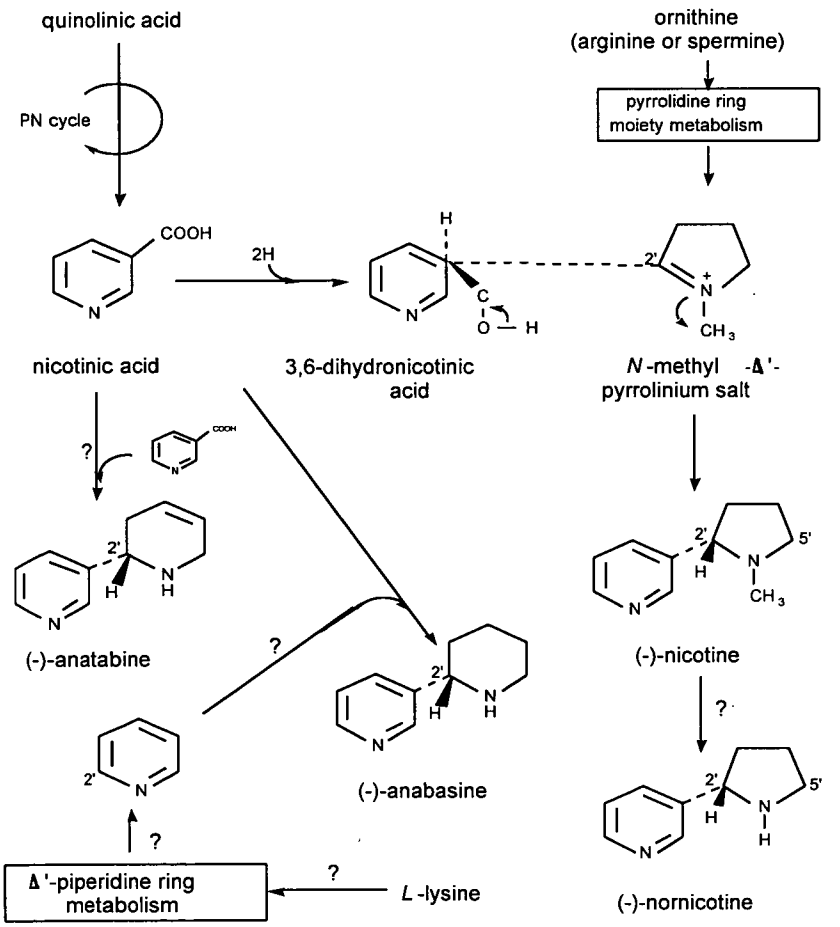


Fig. 1.1. Biosynthesis of Nicotine, Nornicotine, Anabasine and Anatabine (generated from Bush, 1981; Leete, 1985 and Friesen, et al, 1992)
PN cycle: pyridine nucleotide cycle.

The commercial value of tobacco and tobacco goods and, on the other hand, the increasing concerns about the effects of smoking on human health over the last few decades, are attributed to the alkaloid content of tobacco (WHO, International Agency for Research on Cancer, 1986). This conflict has led to many diverse investigations into tobacco and its alkaloid metabolism. For this reason, detailed studies on the metabolism of tobacco alkaloids in tobacco cell cultures were started in the mid-1980s in this laboratory. In this research, attention was concentrated on the bioconversion of nicotine to nornicotine.

1.3. Significance of Study on Nicotine Bioconversion

There are at least FOUR reasons which can be put forward to justify the pursuit of studies on the bioconversion of nicotine to nornicotine. These are:

(1) *To improve the quality of tobacco and tobacco products*

It is well known that the demethylation of nicotine to nornicotine in tobacco leaves during the course of air curing and fermentation is a natural phenomenon of commercial interest (Waller & Nowicki, 1978). In the early years, this conversion, which also reduces the alkaloid content of tobacco, was considered advantageous, since nornicotine is less toxic than nicotine and the transfer of nornicotine into the smoke is less than one-quarter of that for nicotine (Griffith, et al, 1955). In contrast to this, the modern concept is that nicotine appears to increase smoking quality and nornicotine decreases it, as nornicotine contributes a harsh and bitter flavour to cigarettes (Smeeton, 1987). In view of the reduction of tar:nicotine ratio, a high

nicotine content in tobacco compensates for the loss of nicotine during the process of tar removal, which has long been a commercial target (WHO, International Agency for Research on Cancer, 1986 and Hoffmann & Hoffmann, 1989).

(2) *To change the constituents of tobacco so as to reduce possible carcinogenicity*

Although, nicotine itself is not genotoxic and there is no laboratory evidence that it is a carcinogen or that it enhances the activity of known carcinogens (Sir Froggatt & Wald, 1989), nicotine (tertiary amine) and nornicotine (secondary amine) present in tobacco can be nitrosylated to produce nitrosamines which are evidently carcinogenic (Mirvish, 1975). Indeed, it is accepted that the increase in a secondary amine, such as nornicotine, in tobacco is likely to lead to a increase in the yield of total nitrosamines, thus the presence of nornicotine is undesirable (Sweeton, 1987 and Sir Froggatt & Wald, 1989). Study on nicotine bioconversion, therefore, may be helpful in the alternation of the constituents of tobacco so as to reduce possible carcinogenicity.

(3) *For use as an agrochemical*

In contrast to its undesirability, nornicotine is an effective insecticide and feeding deterrent to most animals, as is nicotine (Budavari, 1989 and Luckner, 1990). Also, *N*-acylnornicotine, a nornicotine derivatives with carcinogenic activity, does have biological activity against some insects (Fannin & Bush, 1992). Therefore, nornicotine may be a useful precursor for the production these useful chemicals through, for example, large scale cell culture systems.

(4) *To benefit theoretical studies*

It is well known that a plant and the cultured cells derived from it are capable of converting a variety of exogenous chemicals (Barz, 1977). However, its mechanisms of conversion are little understood. Demethylation occurs very commonly in most living organisms and is involved in many important aspects of metabolisms, such as detoxilations, dealkylations and other reactions against xenobiotics (Fonne-Pfister, et al, 1988). These reactions are either similar or related to each other, being catalysed by a common enzyme complex (West, 1980). Therefore, an understanding of the mechanism of nicotine demethylation could benefit our understanding of other reactions and their biotechnological applications.

1.4. Bioconversion of Nicotine to Nornicotine

1.4.1. Terminology

Throughout the literature, the terms biotransformation, bioconversion, biodegradation and bioturnover have been used to describe similar procedures by different authors. Some of these have gained a new significance through developments in this field. Kieslich (1984) suggested that they should be distinguished and put forward the idea that biotransformations should be considered as selective, enzymatic modifications of defined pure compounds into defined final products. However, secondary products may not always be defined as end products, because they may be further metabolised or degraded. In the absence of specific knowledge of the reactions involved, such further metabolism can be defined as bioturnover (Barz & Köster, 1981). Nevertheless, the proposition that a biotransformation is the enzymatic conversion of an added precursor by cultured

cells (Yeoman, et al, 1990) seems to be more rational as the reactions can be either single-step (mediated by the action of one enzyme) or multi-step (mediated by two or more enzymes).

In some cases, it is impossible within a single term to draw a clear-cut distinction between individual categories, although the term biotransformation has been used by most people. Today, the general goals of biotransformations may be considered to be either the specific modification of substrate structures via selective transformation reactions, or the partial degradation of a substrate into desirable metabolites by means of reaction pathways, even the extension of the substrate structure by the use of biosynthetic reactions to give artificial structures (Kieslich, 1984).

For some specific reactions, employment of biochemical terms, such as *N*-demethylation, could be the best way to describe the biotransformation process. Moreover, *N*-demethylation embraces two general mechanisms, transmethylation and oxidative demethylation (Poulton, 1981). Even so, this term appears to be more precise than biotransformation, in the particular case of the bioconversion of nicotine to nornicotine, even if the mechanism is still unknown.

1.4.2. Literature review

Demethylation and the consequent loss of nicotine in tobacco leaves during air curing and fermentation (Valleau, 1949 and Wada, 1956) has long been a natural phenomenon of commercial importance. It was probably first reported about 50 years ago by Dawson (1945a) that nicotine translocated from the roots of *N. tabacum* was demethylated to nornicotine in an aerial graft. It is now known that this process occurs primarily in the leaves of tobacco, although the roots and stems of certain

Nicotiana species are also active (Poulton, 1981). It has also been shown that higher demethylation activity was found in homogenates from senescing leaves rather than young ones (Waller & Nowicki, 1978). In addition, nicotine demethylation was not only found specific to tobacco, but also found in some other members of the family Solanaceae which normally lack nicotine, such as tomato (Tso & Jeffrey, 1959). In *Nicotiana tabacum*, nicotine demethylation seems to be stereospecific, the unnatural (+)-nicotine being demethylated faster than the natural (-) form and the nornicotine derived from optically pure (-)-nicotine being partially racemized (Kisaki & Tamaki, 1961a). In addition, the administration of nicotine to nornicotine-containing species was found to be coupled with oxygen uptake (Waller & Nowicki, 1978), presuming that this reaction is oxidative(Waller & Nowicki, 1978 and Poulton, 1981).

It can be seen from these reviews that much of the experimental data on this reaction remains controversial, since nicotine *N*-demethylation during the air curing and fermentation of tobacco leaves is a very complex process involving microorganisms. For this reason, caution must be taken in the interpretation of these data when considering this reaction (Alworth & Rapoport, 1965 and Bush, 1981). Despite this continuing interest, the mechanism of this demethylation has not been elucidated since it was first reported 50 years ago. However, the results obtained to date have led to several hypotheses on the mechanism of nicotine *N*-demethylation, which are reviewed in the next section.

1.5. Mechanism of Nicotine *N*-Demethylation

There are two possible explanations for the mechanism of nicotine *N*-demethylation, one is a transmethylation mechanism and the other a *N*-demethylation.

1.5.1. The transmethylation hypothesis

In early studies, the bioconversion of nicotine to nornicotine was regarded as a transmethylation, in which the methyl group of the nicotine molecule was transferred to some acceptor(s) leaving nornicotine as the product (Dawson, 1945b). To support this view, Bose et al (1956) reported that crude homogenates of *N. tabacum* were capable of catalysing the conversion of nicotine to nornicotine in the presence of the methyl group acceptor ethanolamine. Stronger evidence was given by Leete and Bell (1959) who showed that after feeding [methyl-^{14}C]-nicotine to a tobacco plant, 90% of the activity was located in the methyl group of the isolated choline. From this, they presumed that the ability of the *N*-methyl group of alkaloids to participate in reversible transformation may be a general phenomenon. In addition, glycine and ethanolamine were also tested for the involvement in nicotine bioconversion activity in leaf homogenates of *Nicotiana tabacum* (James, 1975). However, more recent evidence does not favour a transmethylation (Waller & Nowicki, 1978) mainly because oxygen uptake parallels the bioconversion of nicotine, suggesting that the reaction may be oxidative. In addition, no convincing methyl group acceptor has been found in responsible for this reaction.

1.5.2. Oxidative *N*-demethylation hypothesis

Apart from the evidence that oxygen uptake is coupled with nicotine demethylation, there is more evidence for an oxidative *N*-demethylation. Kisaki & Tamaki (1961a) found that the nornicotine derived from optically pure (-)-nicotine by demethylation in the scions of *N. tabacum* and *N. glutinosa* grafted onto tomato roots was partially racemized. This racemization apparently occurred during the demethylation, since

optically unchanged (-)-nornicotine was recovered after (-)-nornicotine was fed to these aerial grafts. Accordingly, Kisaki & Tamaki (1961a) made a preliminary assumption that the pyrrolidine ring of nicotine opened during the conversion to nornicotine. Ten years later, this view was accepted and developed by Leete and Chedekel (1974) who produced the hypothesis shown in Fig.1.2 which accounts for the partial racemization of the nornicotine derived from (-)-nicotine.

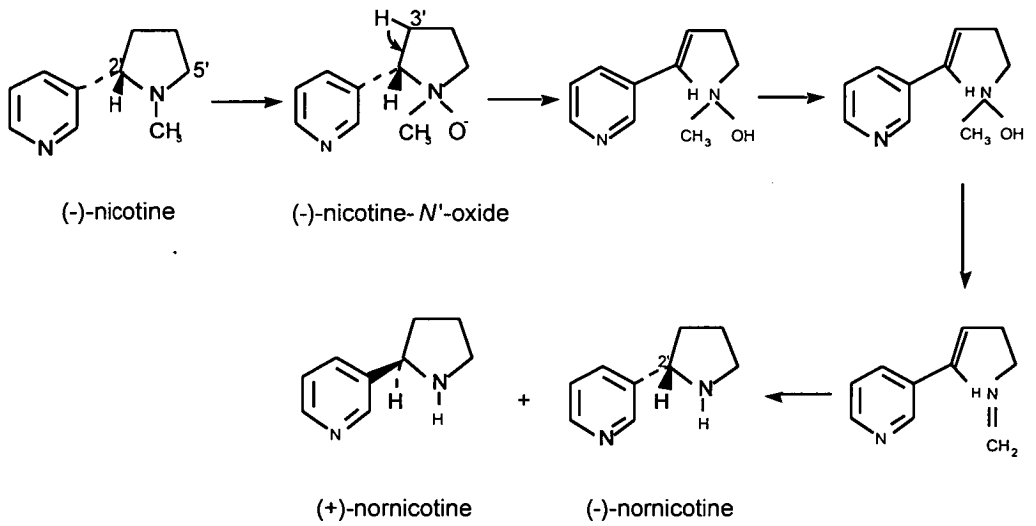


Fig.1.2. Hypothesis for the Formation of (+)- and (-)-Nornicotine from (-)-Nicotine (from Leete & Chedekel, 1974)

However, the proposed intermediate, nicotine-*N*'-oxide, appeared to be unlikely involved in this reaction, because the radioactivity of labelled nicotine-*N*'-oxide fed to tobacco aerial grafts was found in nicotine exclusively rather than nornicotine (Waller & Nowacki, 1978). Another aberrant result was observed by Leete and Chedekel (1972) in which the (-)-nornicotine resulting from C/H-2' labelled nicotine retained most of the original hydrogen at chiral C-2' whereas the (+)-nornicotine lost the hydrogen from the C-2' during the demethylation. This could hardly be explained by the ring-open hypothesis. A few years later, Leete (1977) produced another

mechanism without invoking the opening of the pyrrolidine ring, in which the racemized nornicotine was derived from (-)-nicotine through the iminium salt (see Fig. 1.3).

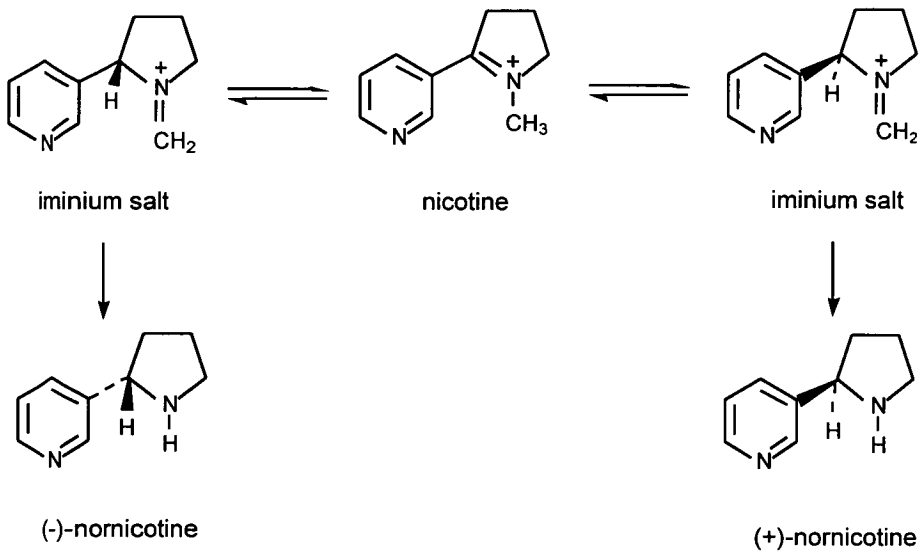


Fig.1.3. Demethylation of Nicotine to Racemic Nornicotine *via* Iminium Salt (from Leete, 1977)

Nevertheless, there has been no convincing evidence to support either of these hypotheses. In view of the yield of a preferential amount of (+) or (-)-nornicotine from (-)-nicotine, it has been presumed that this process is likely to be enzyme-controlled. In fact, there has been an increasing amount of evidence implicating the participation of an enzyme in this *N*-demethylation.

1.5.3. Enzymatic *N*-demethylation

In 1955, Griffith, Valleau and Stokes showed in a breeding study with *Nicotiana tabacum* that the *N*-demethylation which takes place during curing was controlled by

a single dominant gene and predicted the presence of an enzyme which catalysed the *N*-demethylation of nicotine to nornicotine. Bose et al (1956) were probably the first to report that crude homogenates of *N. tabacum* were capable of catalysing the conversion of nicotine to nornicotine. Later, Schröter (1966) also detected nicotine demethylation activity in extracts of *N. alata*. However, both of these reports did not include any details of the enzyme catalysing this reaction. It is only very recently that the enzyme catalysing nicotine *N*-demethylation was demonstrated in a microsomal preparation from *N. otophora* and was partially characterised (Chelvarajan, et al, 1993). This was the first time, it was suggested that nicotine *N*-demethylation may be associated with the enzyme complex of cytochrome P-450.

Cytochrome P-450 is a membrane-bound heme-containing enzyme complex which mediates a wide variety of oxidative reactions (mainly studied to date are hydroxylations or oxidative demethylations) in plant tissues and are involved in the synthesis of a large number of secondary metabolites (Donaldson & Luster, 1991). This protein complex from plant sources is usually located in microsomal preparations, although its function probably differs depending on its subcellular location (West, 1980). *N*-Demethylase has been shown to be linked with cytochrome P-450 in avocado pear (*p*-chloro-*N*-methylaniline *N*-demethylase, Dohn & Krieger, 1984 and O'Keefe & Leto, 1989), artichoke tuber (aminopyrine *N*-demethylase, Fonne-Pfister, et al, 1988) and wheat (chlorotoluron *N*-demethylase, Mougin, et al, 1990). To prove the involvement of cytochrome P-450 in a particular reaction, a number of criteria must be established (West, 1980 and Donaldson & Luster, 1991). These include:

(1) The presence in the reduced enzyme preparation of a CO-binding pigment with a maximal absorbance at 450nm in the CO-difference spectrum;

(2) Inhibition of the reaction in the presence of CO and O_2 in the gas phase when compared with an equivalent concentration of O_2 alone;

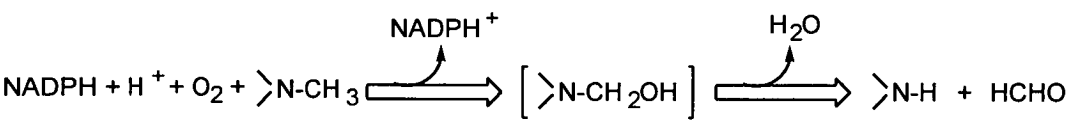
(3) Reversal of CO inhibition by light with a maximum in the photoaction spectrum at 450nm;

(4) Demonstration of the expected reaction stoichiometry, that is, one mole each of NADPH, O_2 and substrate utilised for each mole of product formed;

(5) The incorporation of one oxygen atom from $^{18}O_2$ into each mole of product.

(6) Other types of evidence have also been used, such as the nature of associated electron transport components and the action of other inhibitors, but these are only meaningful when accompanied by the primary evidence already cited.

Accordingly, a typical oxidative demethylation is initiated by hydroxylation following by the non-enzymatic elimination of the hydroxylated methyl group as an aldehyde:



However, the enzymatic study of nicotine *N*-demethylation is currently at a preliminary stage and a more extensive examination is therefore required before the involvement of cytochrome P-450 can be implicated.

1.6. Nicotine *N*-Demethylation in Tobacco Cell Cultures

It is commonly accepted that plant cell culture systems are important in both metabolic studies and the commercial production of useful chemicals (Fowler, 1987). It is evident from the research conducted over the last decades that cell cultures offer many advantages over plants for the elucidation of biosynthetic pathways, such as the absence of microorganisms and the ease of manipulation of cells with phytohormones and various other molecular effectors (Barz, 1977). Despite these advantages, however, nicotine *N*-demethylation has rarely been studied in tobacco cell cultures.

Before the work of Barz, Kettner and Hüsemann (1978), the *N*-demethylation of nicotine to nornicotine had never been studied in tobacco cell cultures, nor had nornicotine ever been isolated as a constituent of nicotine producing cell cultures. In the experiments of Barz et al (1978), it was shown that *N*-demethylation of nicotine to nornicotine by cell cultures of *N. tabacum* and *N. glauca* depended to a varying degree on the auxin level. Failure of callus and plantlets of some tobacco strains which accumulate nornicotine to transform added nicotine to nornicotine (Saunders, et al, 1981) was probably due to an inappropriate phytohormone level or to the culture line employed. Conversely, cell cultures of *N. plumbaginifolia*, a cell line free of nicotine, were able to convert added nicotine to nornicotine albeit at very low conversion rate (Manceau, et al, 1989).

Callus cultures of *N. tabacum* cv. Wisconsin-38, formerly a major source of commercial tobacco, have been shown to be a good system for the study of the mechanism of nicotine *N*-demethylation, due to their ability to accumulate nornicotine (Tiburcio, et al, 1985). This was the reason that Hobbs (1989) established and optimised a cell suspension culture in this laboratory several years ago. Using this cell line, an extensive kinetic study on *N*-demethylation of added nicotine to

nornicotine was carried out (Hobbs & Yeoman, 1991a), showing that the process was probably intracellular and that light caused a slight inhibition to nicotine *N*-demethylation. The results of Hobbs and Yeoman (1991a) contrasted with that of Manceau et al (1989) with cell cultures of *N. plumbaginifolia*, who reported an aberrant kinetic pattern for this conversion, with no correlation between the disappearance of nicotine and the appearance of nornicotine, and that light promoted *N*-demethylation.

It was in attempt to reconcile these differences and to study in detail the mechanism of this nicotine *N*-demethylation which prompted this study.

1.7. Aims and Objectives

The aim of this project was to determine the mechanism of the *N*-demethylation of nicotine to nornicotine in cell suspension cultures of *Nicotiana tabacum*, paying particular attention to the enzyme(s) which catalyse this reaction. The following objectives were pursued towards the fulfilment of this aim:

(1) To investigate the fate of ^{14}C-nicotine and ^{14}C-nicotine added to the cell cultures and to establish the early stage kinetics of this reaction;
(2) To examine the stereospecificity of the nornicotine converted from added nicotine;
(3) To establish whether an enzyme catalysed this *N*-demethylation and to develop an *in vitro* isotopic enzyme assay procedure;
(4) To characterise the enzyme in crude preparations and partially purified enzyme preparations;

(5) To study the enzymology of the reaction, its subcellular localisation and the effect of light on enzyme activity.

Chapter Two:

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant material

A mixotrophic cell suspension culture of *Nicotiana tabacum* cv. Wisconsin-38, established and optimised in this laboratory approximately 5 years ago (Hobbs, 1989), was used throughout this investigations.

2.1.2. Authentic standards

Highly purified standards of (-)-nornicotine ([-]-2'-[3'-pyridyl]-pyrrolidine) (>98.5%) and (±)-nornicotine ([±]-2'-[3'-pyridyl]-pyrrolidine) (>91.5%) were supplied by Rothmans International Services Ltd. (-)-nicotine ([-]-1'-methyl-2'-[3'-pyridyl]-pyrrolidine) (98%) and (±)-nicotine ([±]-1'-methyl-2'-[3'-pyridyl]-pyrrolidine) (99%) were purchased from Sigma. The sources of other chemicals employed in the experiments described in this thesis are indicated individually.

2.2. Cell Suspension Cultures

2.2.1. Preparation of Culture Medium

Liquid Gamborg's B_5 medium (Gamborg et al, 1968) supplemented with sucrose (30g/l), NAA (0.15mg/l), kinetin (0.2mg/l) and ascorbate (5mg/l) was used as the standard growth medium. It was prepared by dissolving 3.874g/l of Gamborg's B_5 basic salts (Imperial Laboratories) in distilled water together with 30g/l of sucrose (Fisons Ltd.) and appropriate amount of growth regulators (Sigma Ltd.). Growth

regulators were dispensed from the stock solutions, at a concentration of 0.15mg/ml for NAA. 0.2mg/ml for Kinetin and 5mg/ml for ascorbate respectively, which were stored in the refrigerator at 4°C after preparation. The pH of the medium was finally adjusted to 5.6 before sterilisation.

2.2.2. Sterilisation

2.2.2.1. Sterilisation by heat

Culture medium was sterilised, after dispensing into conical flasks which were then sealed with two layers of aluminium foil, by autoclaving at 121°C and 15p.s.i. for 20min.

2.2.2.2. Sterilisation by filtration

For feeding experiments, stock solution of nicotine was sterilised by passing through a 0.22μm filter held in a Sterivex-GS filter unit (Millipore), prior to the aseptic addition to cultures.

2.2.3. Cell suspension culture

2.2.3.1. Culture conditions

Cell suspension cultures were routinely grown in the dark, at a temperature of 25±1°C, on a rotary-shaker at a speed of approximately 90rpm and 10mm amplitude.

2.2.3.2. Subculture and maintenance of cell suspensions

Subculture of cell suspension cultures were carried out every 20 days, by the aseptic transfer of 1g fresh weight of cells to 50ml of fresh sterilised medium in a 250ml conical flask. After sealing with two layers of aluminium foil, the flasks were incubated on the rotary-shaker under the condition described in 2.2.3.1. This procedure was also used to maitain suspension cultures.

2.3. Characterisation of Culture Growth

Three parameters were selected to characterise the growth of cell suspension cultures. They were packed cell volume (PCV), fresh weight and dry weight.

2.3.1. Determination of packed cell volume (PCV)

The packed cell volume (PCV) of cell suspension cultures was measured using the method of Reinert & Yeoman (1982). This was achieved, at each sampling time, by centrifuging all of the suspension culture in each 250ml flask using a 50ml graduated centrifuge tube at 1000*g* for 5min. The percentage of the cell pellet volume expressed against the total volume is the Packed Cell Volume.

2.3.2. Determination of fresh weight

Following the measurement of PCV as in 2.3.1, fresh weight of the cell suspension culture was determined after removal of the medium by filtration under reduced pressure through miracloth (Calbiochem) using a Büchner funnel. The resulting cell mass was weighed and defined as fresh weight.

2.3.3. Determination of dry weight

The cell mass after fresh weight measurement (see 2.3.2) was washed three times with distilled water to remove the residual sucrose and then dried in an oven at 80°C for 24hr. After cooling in a desiccator, the dried cell mass was weighed and defined as dry weight.

2.4. Feeding Experiments

2.4.1. Non-radioactive nicotine feeding experiments

For standard feeding experiments, a stock solution of nicotine, 10mg/ml (62.5μmol approx.), was prepared in advance by dissolving the appropriate amount of (-)-nicotine (*1'*-methyl-*2'*-[*3'*-pyridyl]-pyrrolidine) (Sigma) in distilled water. 1ml of this stock solution was then added to each of 250ml conical flasks containing 10 day old cell cultures.

2.4.2. Radioactive nicotine feeding experiments

5μl (0.5μCi, 9.5×10^{-3}μmol) of *dl*-(2'-^{14}C)-nicotine (*NEN*, Du Pont) was added aseptically with a microsyringe to each of 50ml conical flasks containing 10ml of a randomized 10 day old cell culture. Further details about the radioactive nicotine are described in 2.6.4.1.

2.5. Extraction of the Tobacco Alkaloids

2.5.1. Extraction for TLC and HPLC meassurements

Suspended cells were separated from the medium by vacuum filtration, through miracloth (Calbiochem), using a Büchner funnel unit. Samples of both cells and medium were extracted separately.

Potassium hydroxide (BDH) was added to the medium to a final concentration of 2M. After hydrolysis for 1hr at 90°C, the medium was extracted three times with an equal volume of chloroform. After removing the dissolved water with anhydrous sodium sulphate, the chloroform extract was filtered, through a glass microfibre filter (Whatman), using a Büchner funnel unit and evaporated to dryness under vacuum at 30°C in a Büchi Rotavapor R-110 system. The residue was resuspended in 1ml of HPLC grade methanol and then filtered through a 0.45μm nylon filter (Whatman), supported in a Swinnex disc filter holder unit(Millipore). The filtrate was .was stored in a 1.5ml brown HPLC sample vial at 4°C until required.

The cells were ground in an approximately equivalent volume of 1N HCl in a pestle and motar. The cell debris was then removed by filtration under pressure and the filtrate was extracted as for medium.

2.5.2. Extraction for GC-MS and optical property analysis

In order to avoid any possible chemical or stereochemical alteration during extraction, a mild procedure was used to extract the alkaloids from cells and medium.

Although the extraction efficiency of this method was not as high as that described in 2.5.1, it was adequate for GC-MS and optical property analysis.

After separation of cells from medium by filtration, the pH of the medium was adjusted to 11 with concentrated amonia hydroxide (35%) and alkaloids in the medium were extracted three times with an equal volume of chloroform. The resulting chloroform extract was treated in the same way as that in section 2.5.1.

Cells was ground up in a pestle and mortar in an equivalent volume of 1N HCl and the cell debris was removed by filtration under vacuum. The filtrate was then treated as for the medium.

2.6. Chemical Analysis of Tobacco Alkaloids

2.6.1. Qualitative analysis by Thin Layer Chromatography

2.6.1.1. Thin layer chromatography (TLC)

Qualitative analysis of tobacco alkaloids in the extracts was carried out using TLC. A plastic-supported TLC plate, coated with silica gel 60 to a thickness of 200μm (Merck), was used. For the separation and purification of a mixture of tobacco alkaloid, a preparative glass-supported PK6F Silica Gel 60A TLC plate with fluorescent indicator (Whatman) was used. The thickness of the plate was 1mm. The solvent system, for either qualitative or preparative TLC, consisted of chloroform:methanol:20% amonia hydroxide in a proportion of 60:10:1.

2.6.1.2. Preparation of the iodoplatinate reagent

The iodoplatinate reagent was prepared as the following precedure: 10ml of a 5% (w/v) platinum chloride solution [made up as 0.5g chloroplastinic acid (Fisons) dissolved in 5ml M HCl plus 5ml distilled water] and 5ml concentrated HCl (BDH) were added to 240ml of 2% (w/v) potassium iodide (BDH) solution.

2.6.1.3. Analysis of samples

Samples, and the corresponding alkaloids standards, in an appropriate concentration were loaded at 1cm to 1.5cm intervals onto the TLC plate 1.5cm above its bottom edge. After drying, the plate was eluted for about 1.5hr in a sealed TLC tank with the solvent system described in 2.6.1.1. The atmosphere in the tank had been equilibrated for 30min in advance with the same solvent. When the solvent front reached a point 2cm from the top edge, the plate was taken out of the tank and dried in a stream of air in a fume cupboard. Then the plate was sprayed with the iodoplatinate reagent in a Humbrol spray gun as described in 2.6.1.2.

Fig.2.1 is a diagram of a typical TLC plate, in which alkaloids were identified by comparison with authentic standards. On a newly eluted and sprayed TLC plate, the alkaloids appear as blue-grey spots on a pink background. The colour of both spots and background soon fades when exposed to light.

For the preparation and purification of alkaloid samples, the alkaloids separated on a preparative TLC plate were detected under UV light at 254nm, at which wavelength the alkaloid spots cause fluorescence quenching.

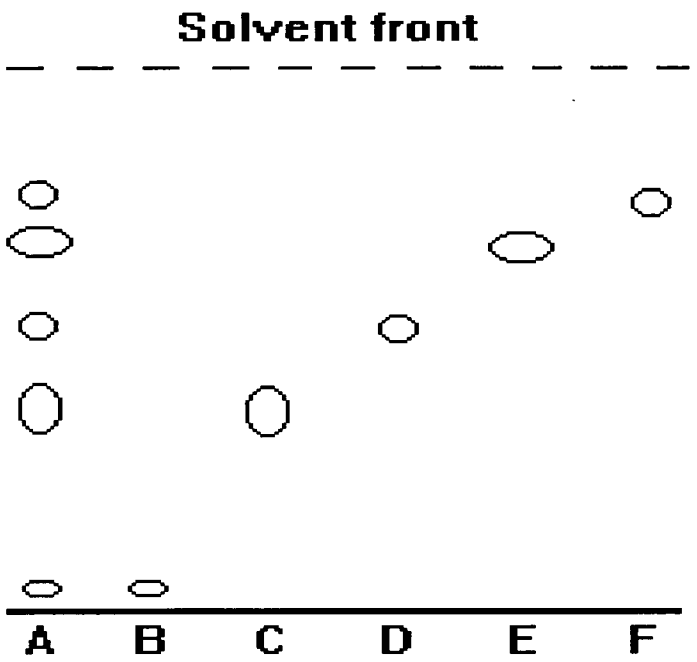


Fig.2.1 A TLC chromatogram Showing Some Authetic Tobacco Alkaloid Standards and Nicotinic Acid Separated and Detected with the Method Described in 2.6.1

A: a mixture of *B*, *C*, *D*, *E* and *F*; *B*: nicotinic acid (2μg); *C*: nornicotine (2μg); *D*: anatabine (2μg); *E*: nicotine (2μg) and *F*: myosmine (2μg).

2.6.2. Quantitative analysis by HPLC

High Performance Liquid Chromatography (HPLC) was used routinely to determine the amount of nicotine and nornicotine in the extracts. HPLC is much more sensitive than TLC and can identify down to 5μg of nornicotine and 1μg of nicotine under the conditions used here (see 2.6.2.1 and 2.6.2.2). This system is ideal for the estimation of very small amounts of nicotine and nornicotine.

2.6.2.1. High Performance Liquid Chromatography system

A Gilson 302 liquid chromatography system was used to perform the quantitative analysis of the tobacco alkaloids. This system consisted of a Gilson Holochrome variable wavelength UV detector, together with a Gilson 231 autosample injector coupled to a Gilson 801 dilutor and ran by an IBM PS-2 computer equipped with 714 HPLC system controller software which, automatically controlled the overall processes from sample injection to data analysis.

A Nova-Pak C18 60Å 4m 3.9 ×150mm column (Millipore Waters) was used to separate the tobacco alkaloids. A mobile phase, consisted of methanol (HPLC grade, BDH) and newly prepared 0.2% (v/v) orthophosphoric acid buffer. The orthophosphoric acid buffer was prepared as follows: 2ml of orthophosphoric acid (BDH) was dissolved into 950ml of HPLC grade water (BDH) and the pH was adjusted with triethylamine (BDH) to 7.5 (Saunders & Blume, 1981). The mixture was then made up to 1000ml with the HPLC grade water (BDH) and filtered under pressure through a 0.2μm pore size of cellulose nitrate filter (Sartorius) held in a 1000ml Millipore vacuum filtering unit.

2.6.2.2. HPLC analysis of samples

Samples in brown HPLC vials were prepared as described in 2.5.1. Both methanol and the buffer were degassed for 15min with helium before starting the measurements, which were made at room temperature, on the Gilson 302 liquid chromatographic system. The samples were automatically injected into the system and eluted, at a flow rate of 1ml/min, with the mobile phase described in 2.6.2.1 employing a gradient between methanol and the orthophosphoric acid buffer (see Fig.2.2). The maximum injection volume was 20μl. Tobacco alkaloids were

separated by the column after a total elution time of 35min and detected at a wavelength of 260nm.

As this system was automatically controlled by a computer, the overall processes, from sample injection to data analysis, were simplified. The amounts of nicotine and nornicotine in the samples were calculated according to the area of the peak with the corresponding retention time of their authentic standards. An HPLC chromatogram of mixed authentic nicotine and nornicotine standards is shown in Fig.2.2.

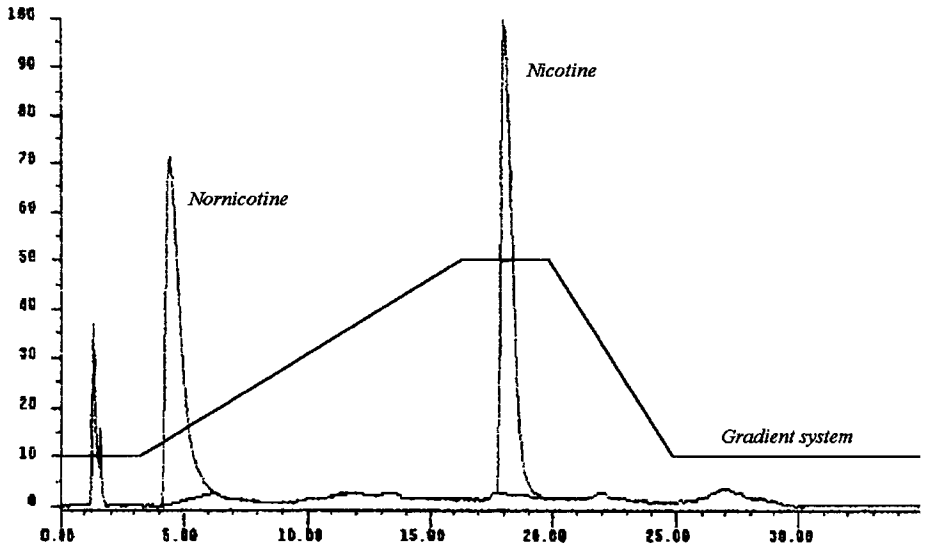


Fig.2.2 An HPLC Chromatogram and the Gradient System Used Showing Clear Separation between Nicotine and Nornicotine Standards

Compounds were separated and detected by the system described in 2.6.2.2.

2.6.3. Determination of the optical property of the tobacco alkaloids

2.6.3.1. Determination by polarimetry

The optical rotation of the nornicotine converted from added (-)-nicotine by the cultures, and the nicotine left after the convertion, were determined at 589nm on a Perkin-Elmer Model 141 polarimeter at room temperature. A standard 1cm sample cell was used. The samples were prepared as described in 2.5.2 and purified by preparative TLC as desrcibed in 2.6.1.3. The purity of the samples in 2ml of methanol were examined on HPLC, as was the concentration of the alkaloids, prior to polarimetric analysis. Before the analysis started, the polarimeter was zeroed using HPLC grade methanol as a blank. Calculation of the specific optical rotation ($[\alpha]_D$) is described in detail in 2.12.5.

2.6.3.2. Determination by Chiral Gas Chromatography

In order to confirm the results obtained from the polarimetry, the optical properties of nornicotine were studied using Chiral GC by Dr. Elmenhorst at Rothmans International Services Ltd, Bremen. After the polarimetric measurement, 6mg of the same nornicotine sample, which had been converted from added (-)-nicotine by the cell cultures, was sent off for analysis.

In the analysis, 6mg nornicotine was dissolved in 0.5ml CH_2Cl_2 and 10mg of (*1S*)-(-) -camphanic acid chloride were added. After reaction for 1h at room temperature, the sample was evaporated in a rotary evaporator to remove the unreacted organics. The resulting *N'*-camphanoyl-nornicotine diastereoisomeric amide derivatives were then injected into a capillary GC and separated on a 30m DB5 wide-bore column, with an oven temperature of 260°C and helium as the carrier gas. If the nornicotine was optical mixture there should be two peaks with very close retention times. Otherwise there would only be one peak.

2.6.4. Measurement of radioactivity

2.6.4.1. Radioactiive nicotine

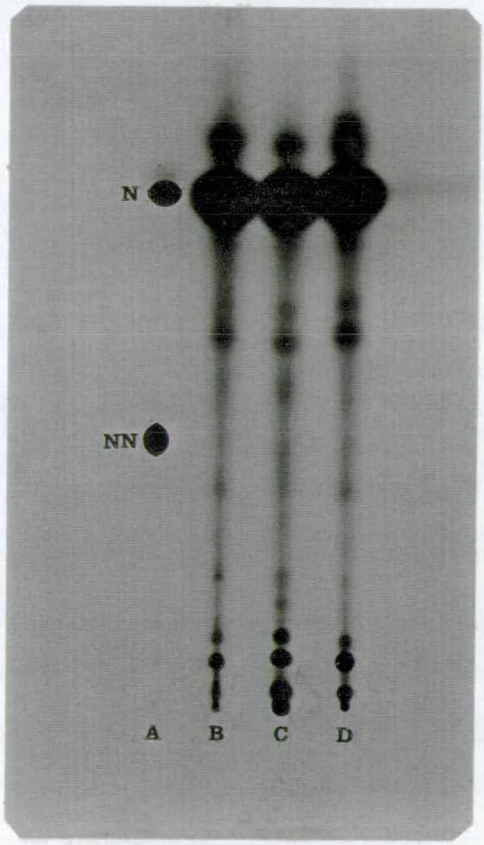


Fig.2.3 An Autoradiograph Showing the Composition of the ^{14}C-Nicotine Supplied by *NEN* Du Pont

A: Cold nicotine and nornicotine standards (both at 2μg); B: Extracts of the control for the enzyme assay (0.2μCi added); C: ^{14}C-Nicotine stock solution prepared with the buffer described in 2.7.1 (0.2μCi loaded) and D: Pure ^{14}C-nicotine from the supplier (0.2μCi loaded).

The radioactive nicotine used in the experiments throughout this thesis was [pyrrolidine-*2'*-^{14}C]-nicotine (M.W. 162.2) supplied by *NEN* Du Pont. The specific

activity was 52.7mCi/mmol (1.9GBq/mmol) and the purity was 97.7% according to the technical data report from the supplier. The purity of this product was checked every two months using either HPLC or TLC as recommended by the supplier. Fig.2.3. is an autoradiograph of a TLC plate showing the quality of the product.

2.6.4.2. Semi-qualitative determination of radioactivity in alkaloids by autoradiography and RITA

Autoradiography was used to locate the radioactive spots on a developed TLC. This was achieved, in the dark at room temperature, by placing an appropriate size of autoradiography film (Hyperfilm-MP, Amersham) onto the TLC plate and putting them into an autoradiographic cassette. The cassette was firmly closed and placed into a -70°C freezer. After about 20 days exposure, depending on the radioactivity of the samples, the cassette was taken out of the freezer and left in the laboratory at room temperature for 1 - 2h prior to processing the film in the dark in a Compact X2 automatic film processor (X-Ograph Ltd., UK).

Radioactivity Intelligent Thin-layer Analyzer (RITA) was used to make rapid measurements of the radioactive distribution of the spots on a developed TLC. The RITA equipment used was an Isomess IM-3000 Radio-TLC Analyzer (Nuclear-Interface) which was controlled, from scanning to data output, by a computer.

2.6.4.3. Quantitative determination by liquid scintilation counting

Quantitative determination of either aqueous samples or scraped silicon gel spots from TLC plates was carried out by liquid scintillation counting with a Beckman LS 1175 Liquid Scintillation Counter. A scintillant cocktail consisting of 1.0L toluene

and 10g butyl-PBD (Malcolme-Lawes, 1979) was used for non-aqueous samples and, for aqueous samples 500ml Triton X100 was added to the cocktail. Prior to counting, the scintillant (usually 5ml), which was about 20 times greater than the sample volume, was added to a 20ml scintillation vial (Zinsser) together with the sample.

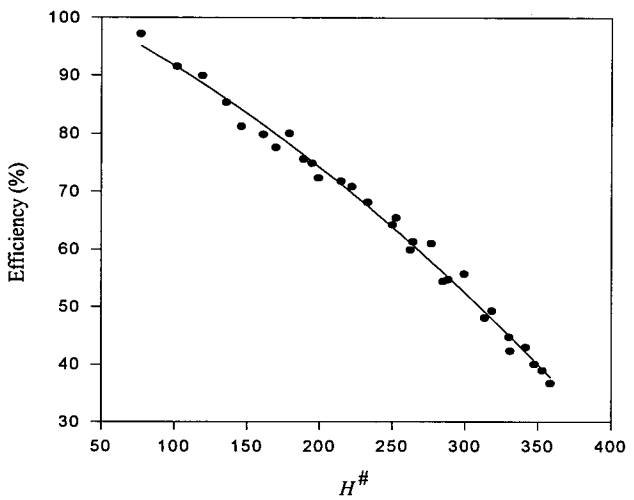


Fig.2.4 A Quench Correction Curve under the Conditions Described in 2.6.4.3.

Before counting the samples, the counting efficiency was estimated. As a result of quenching, the procedure of estimating the efficiency is referred to as the estimation of the quench correction. To achieve this, a known radioactivity (i.e. 9850dpm) of ^{14}C-toluene (Amersham) was quenched by the addition of afferent amount of acetone (quench reagent) and then counted. Two groups of values were obtained, one was cpm (counts per minute) and the other was $H^{\#}$ value which was expressed as the reciprocal of machine efficiency. The quench efficiency was then calculated as a percentage of quench (cpm) to unquench (known dpm, disintigrations per minute). The quench correction curve shown in Fig.2.4 was obtained by plotting the $H^{\#}$ values

against the quench efficiency. Using this curve, the counting efficiency of each sample could be determined.

Alternatively, for the sake of convenience, an equation was produced, by regressing between $H^{\#}$ values and the quench efficiency of the correction curve, using a computer statistical package (MINITAB, USA). A determination coefficient of this repression was achieved as high as 99.6%. The equation is shown below:

$$E = 96.8 + 0.0618\ H^{\#} - 0.000639\ H^{\#2}$$

Where: E = percentage quench efficiency;

$H^{\#}$ = the reciprocal of machine efficiency.

2.6.5. Identification of unknown compounds by GC-MS

2.6.5.1. Separation and preparation of the samples

It can be seen from Fig.3.11 that several radioactive labelled compounds, other than nicotine and nornicotine, appeared on the TLC plate in both the cells and the medium 6hr after the addition of ^{14}C-nicotine to a 10 day old culture, although nornicotine was the main product of the added nicotine. Some of these labelled compounds Which situate below the unknown compound (UC) spot (see Fig.3.11) can be accounted for by the impurities of the ^{14}C-nicotine (compare Fig.3.11 with Fig.2.3). In order to determine whether any of these unknowns were intermediate(s) of the bioconversion of nicotine to nornicotine, 10mg of cold nicotine (Sigma) was added to a 250ml conical flask containing 50ml of a 10 day old cell culture. This culture was incubated for 48hr under the conditions described in 2.2.3. Subsequently, the

alkaloids in both cell fraction and medium were extracted, using the method described in 2.5.2, and separated by preparative TLC (see 2.6.1). The spots corresponding to the unknowns were marked on the TLC plate by referring to the co-eluted 48h radioactive sample from 3.4.2. The silicon gel of the unknowns on the TLC plate was then scraped and washed with HPLC grade methanol. The volume of the extracts in methanol was reduced to 1ml under vacuum by evaporation at 30°C and filtered through a Millipore filter with a pore size of 0.45μm prior to GC-MS analysis.

2.6.5.2. GC-MS analysis of Samples

Sample analysis was performed on an HRGC-MS System (KRATOS MS 50TC), with a 50m x 0.32mm I.D. fused silica capillary column (CP-Sil 5) and 10% of helium gas as eluent, and a temperature programme of 70 - 100°C at 3°C/min and 100 - 250°C at 5°C/min. Electron impact mass spectra (70eV) were recorded with a source temperature of 210°C. All metabolites were identified by reference to their GC retention time and the MS spectra of authentic standards.

2.7. Assay of the Enzyme(s) Catalysing the Bioconversion of Nicotine to Nornicotine

2.7.1. Preparation of the buffer system

The buffer system developed for the assay of the enzyme(s) catalysing the bioconversion of nicotine to nornicotine was 0.1M Tris-HCl buffer (pH 9.0) supplimented with 3mM DTT and 5mM EDTA. This buffer and the additives were

all prepared with double-distilled water and then chilled in a refrigerator at 4°C prior to the preparation of cell-free enzyme extracts.

2.7.2. Preparation of cell-free enzyme extracts

All processes were carried out in a cold room at 4°C. After separation of the cells from the medium by filtration under reduced pressure, the 10 day old cell cultures were homogenised with the buffer described in 2.7.1 at an 1:1 (w/v) ratio of cell:buffer, with acid washed sand, in a pestle and mortar. The homogenate was subsequently filtered through two layers of muslin and clarified by centrifugation at 8,800*g* for 1min on a Eppendorf Centrifuge 5413. The supernatant was then used for enzyme assay. A schematic flow chart of this procedure is illustrated in Fig.2.5.

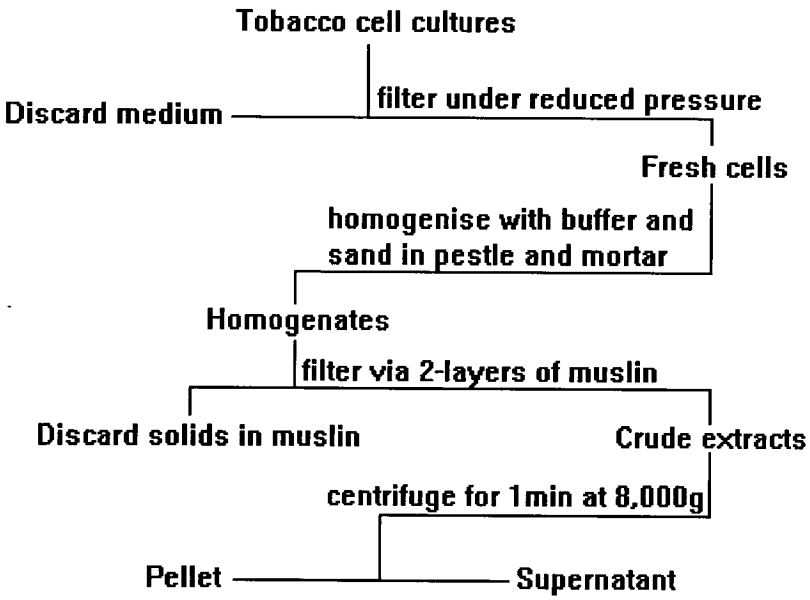


Fig.2.5 Schematic Flow Chart Describing the Major Steps for the Preparation of a Cell-free Enzyme Extract

2.7.3. Enzyme assay

The activity of the enzyme(s) catalysing the *N*-demethylation of nicotine to nornicotine was examined by measuring the radioactivity of ^{14}C-nornicotine produced from added ^{14}C-nicotine. The reaction was started by the addition of 10μl of ^{14}C-nicotine (*NEN*, Du Pont) stock solution, which was corresponding to 3.8×10^{-3} μmoles (0.02μCi), to a volume of 0.5ml of enzyme extract contained in a 1.5ml Eppendorf centrifuge microtube. After incubation at 30°C in an Eppendorf Thermostat 5320 for 30min., the reaction was terminated by the addition of three drop of concentrated ammonia solution (35%) and an 1ml of chloroform to each of the reaction tubes. The tubes were subsequently shaken with an Eppendorf mixer 5432 for 5min and the border between the organic and aqueous phases was clarified by centrifugation on an Eppendorf centrifuge 5412 for 1min. The chloroform phase was then separated by pipetting and the aqueous phase was re-extracted for two more times with equal volumes of chloroform. The three chloroform fractions were finally combined and evaporated in an air stream to a final volume of 0.1ml.

The control for the assay was processed as for the experimental treatments, but was heated to denature the enzyme(s) in a water bath at 100°C for 5min.

Using this extraction procedure, at least 91% of the total added radioactivity (0.2μCi, 4.44×10^5dpm) was recovered in the chloroform phase (mean of 10 replicates tested).

10μl of concentrated chloroform extract from each of the reaction tubes and control tubes were loaded onto a Silica Gel 60 F_{254} TLC plate, together with 0.2μg of cold authentic nicotine (Sigma) and nornicotine (Rothmans) as carriers and markers, and eluted using the method described in 2.6.1. After drying, an autoradiograph was

prepared as described in 2.6.4.2. Alternatively, each alkaloid spot on the TLC plate was marked with a pencil under UV light and, then, scraped off and counted as described in 2.6.4.3.

All experimental values were expressed as the mean±standard deviation of three replicates, after subtracting blank values which only contained scintillant.

2.7.4. Protein assay

Protein concentration was estimated using the method of Bradford (1976). The protein reagent was prepared by dissolving 100mg of Coomassie Brilliant Blue G-250 (Sigma) in 50ml of 95% (v/v) ethanol. To this solution, 100ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. 2.0mg of Brovine serum albumin (Sigma) was dissolved in 2ml of the same buffer solution as employed in enzyme assay and used to prepared a calibration curve.

The solution containing 10 to 100μg protein in a volume up to 0.1ml was pipetted into a 6ml disposable cuvette (Elkay Ultra-Vu). The volume in the cuvette was then adjusted to 0.1ml with the same buffer as the enzyme assay, 5ml of protein reagent added and the contents mixed thoroughly. After allowing a reaction time of 5 to 10 min, the absorbance at 595nm was measured against a reagent blank which contained 0.1ml buffer and 5ml of protein reagent. The amount of protein (μg) was plotted against the corresponding absorbance resulting in a standard curve (as seen in Fig.2.6) which was then used to evaluate the protein content of unknown samples. A protein weight range from 1 to 50μg was selected for all protein assays, since this is within the range where the protein dye binding response is linear.

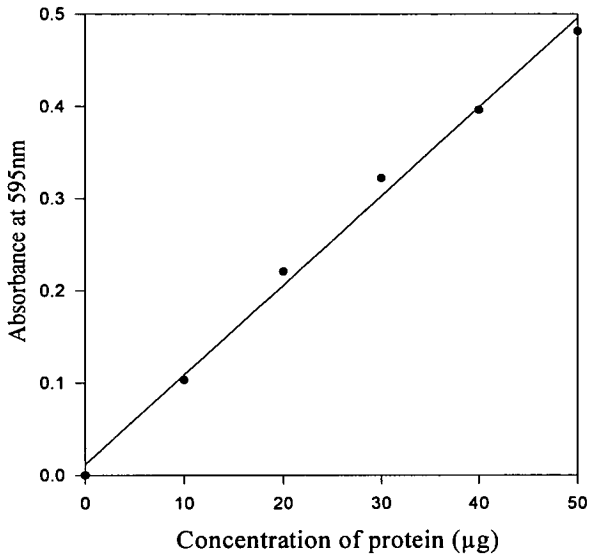


Fig.2.6 A Protein Calibration Curve Using BSA

For the sake of convenience, the relationship between μg protein and the corresponding absorbance at 595nm in the standard curve was incorporated into an equation using a computer package (Minitab, USA). The regression coefficients achieved were as high as 99.4%. Therefore, it was very convenient using this equation to work out the protein content of unknown samples. The equation is shown below:

$$P = 103A - 1.05$$

where: P = protein in μg;

A = absorbance at 595nm.

2.8. Subcellular Fractionation of Nicotine *N*-demethylase

A 7 day old cell culture was used to investigate the subcellular distribution of Nicotine *N*-demethylase. This was a compromise between the maximum activity of this enzyme(s) reached in a culture cycle (see Fig.3.22) and the availability of biomass. All processes were carried out in a cold room at 4°C.

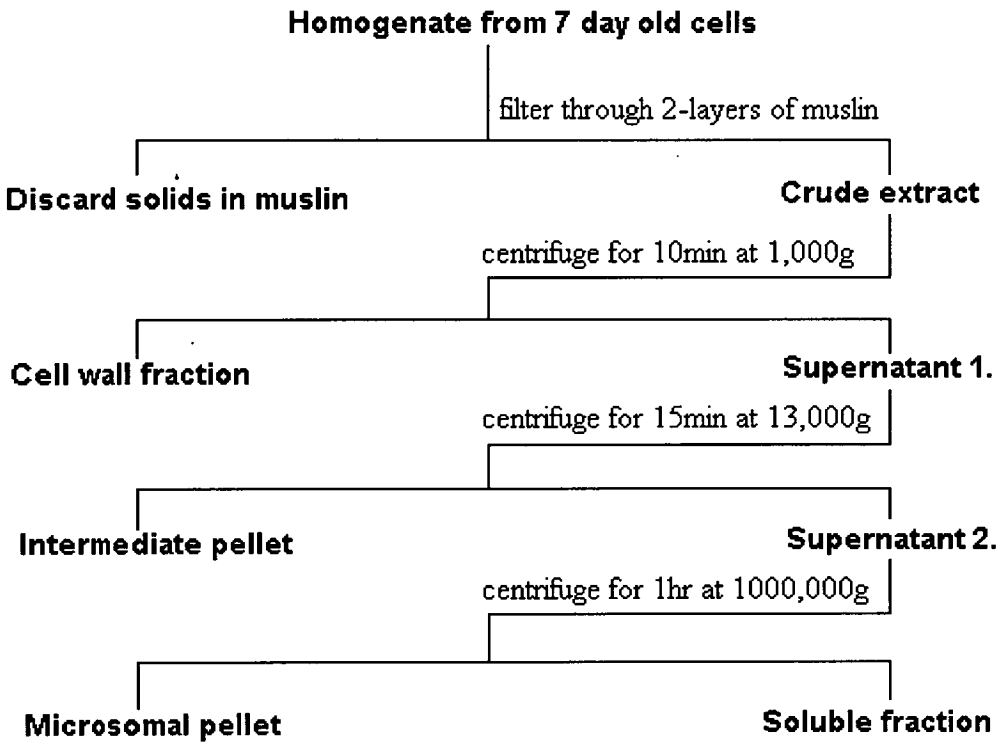


Fig.2.7 Schematic Flow Chart Showing the Major Steps Involved in the Preparation of an Enzyme Extract and its Subcellular Fractionation

After separation of the cells from the medium by filtration under reduced pressure, approximately 10g of the 7 day old cell culture was homogenized, together with acid washed sand, in 15ml of 0.1M Tris-HCl buffer (pH9.0) supplemented with 2mM DTT, 5mM EDTA and 0.25M sucrose in a pestle and mortar. The homogenate (*ca.* 22ml) was first strained through two layers of muslin and centrifuged at 1,000*g* for 10min to obtain the wall pellet. The supernatant (S1) was then centrifuged at 13,000*g*

for 15min to obtain the intermediate pellet. Both centrifugations were made using an RC-5B Sorvall Centrifuge with an SS-34 superspeed angle rotor (Du Pont). Subsequently, the supernatant (S2) resuling from the second centrifugation step was centrifuged at 100,000*g* for 1hr, in an OTD-55B Sorvall Centrifuge with a T865.1 ultraspeed angle rotor (Du Pont), to give soluble fraction (S3) and the pellet. All pellets from each centrifugation were resuspended in 2ml of the same buffer as used in the enzyme preparation. Finally, 0.5ml of each of these resuspended pellets and the supernatants were assayed for both protein and nicotine *N*-demethylase as described in 2.7.3 and 2.7.4. A schematic flow chart of this procedure is presented as Fig.2.7.

2.9. Preparation of Subcellular Fractions for Transmission Electron Microscopy (TEM)

To investigate the ultrastructural composition of the subcellular fractions, the four fractions obtained from 2.8, i.e., intact cells, cell wall pellet, intermediate pellet and microsomal pellet, these were examined by TEM. The preparation and examination of the samples was performed under the supervision of Mr John Findlay at the Science Faculty, Electron Microscope Facility, University of Edinburgh.

Specimens of the four fractions were first fixed in 2% glutaraldehyde in 50mM sodium cacodylate buffer at pH7.2 for approximately 30min and then washed three times at 20min interval with 50mM sodium cacodylate buffer, prior to post-fixation with 1% osmium tetroxide for about 2hr. After washing three times in distilled water, the specimens were dehydrated according to the following schedule:

Reagent	Time (min)
20% ethanol in water	20
40% ethanol in water	20
60% ethanol in water	20
70% ethanol in water	20
80% ethanol in water	30
90% ethanol in water	60
100% ethanol	60
100% dry ethanol	240
100% dry ethanol	overnight

The specimens to be embedded in resin (the resin was Agar100 resin, an Epon812 substitute which was made up in the following mixture: resin : DDSA:MNA:BDMA = 50:40:20:2, v/v/v/v, Agar Scientific Ltd.) were infiltrated through propylene oxide, 100% dry ethanol and resin in the following ratios (v/v/v):

Propylene oxide	100% dry ethanol	Resin	Time (hr)
1	2	---	0.5
1	1	---	0.5
2	1	---	0.5
3	---	---	1.0
2	---	1	1.0
1	---	1	1.0
1	---	2	2.5
---	---	3	4.0
---	---	pure	overnight

Subsequently, the specimens were embedded in resin in beem capsules and polymerised in an oven at 60°C for 24hr.

In the next stage, the polymerised specimen blocks were trimmed and sectioned on a Reichert-Jung ultramicrotome (Austria). The resulting sections were picked up on formvar coated grids (200 mesh, Agar Scientific Ltd.) and then stained in a two-step procedure as:

Firstly, 4% uranyl acetate (aqueous) for 2hr and washed thoroughly with distilled water;

Secondly, Reynolds lead citrate (Reynolds, 1963) for 6min determined empirically and washed thoroughly with distilled water and air-dried.

Finally, all sections were examined on a Jeol 100s TEM (Japan). Photos were taken on Kodak SQ163 TEM film which was subsequently developed for 4min in D19 developer as recommended by the manufacturer (Kodak).

2.10. Dialysis of the Enzyme Preparation

All processes were carried out in a cold room at 4°C. A 7 day old culture was homogenized with the buffer described in 2.7.1 at a ratio of 1:1 (w/v) of cells/buffer, with acid washed sand, in a pestle and mortar. The homogenate was then filtered through two layers of muslin and centrifuged at 1,000*g* for 5min in an RC-5B Sorvall Centrifuge with an SS-34 superspeed angle rotor (Du Pont). The supernatant was subsequently transferred to a 5×24/32" tube of semipermeable membrane (Medicell International Ltd.) and both ends sealed firmly, prior to dialysis overnight against the same buffer used for the enzyme preparation. A ratio of enzyme solution to buffer of

1:100 (v/v) was used. Finally, the dialysed solution within the membrane was used as a source of the enzyme.

2.11. Preparation of a Sephadex G-25 Gel Filtration Column

In order to separate nicotine *N*-demethylase from the small molecules which might be involved in the bioconversion of nicotine, a Sephadex G-25 gel filtration column was selected. To prepare the column, 3*g* of Sephadex G-25-150 (Sigma) with a bed volume of 4-6ml/*g* was first hydrated at room temperature with 30ml double distilled water for 3hr while gently stirring with a glass rod. The hydrated gel slurry was then poured with care to avoid generating bubbles into a vertically mounted 20×1cm glass column. With natural sedimentation, the gel particles produced an effective gel height of 10cm. A 20cm column extender was connected to the top of the gel column to achieve a gravitational flow rate of approximately 2ml/min. Finally, the column was moved to a cold room (4°C), where it was equilibrated for approximately 2hr with a chilled 0.1M Tris-HCl buffer (pH9.0) supplemented with 5mM EDTA and 3mM DTT, before the application of the samples.

2.12. Definitions and Mathematical Calculations of Data

2.12.1. Room temperature

'Room temperature' was 20±5°C in the laboratory where most of the experiments were carried out unless otherwise stated.

2.12.2. 10 Day old cells

As most of the investigations reported in this thesis was performed using 10 day old cells, it is therefore necessary to define '10 day old cells', i.e., a cell suspension culture incubated, under the conditions described in 2.2, for 10 days after the aseptic transfer of 1*g* cells at the end of subculture (20 days) to a 250ml conical flask containing 50ml freshly prepared medium.

2.12.3. Percentage bioconversion

(1) For nicotine feeding experiments:

The percentage bioconversion is defined as the amount of nornicotine produced, expressed as a percentage of the amount of nicotine added to the culture after taking account of endogenous alkaloids.

(2) For radioactive nicotine feeding experiments:

The percentage bioconversion is defined as the total amount of radioactivity in the nornicotine spot on the TLC plate, expressed as a percentage of the total radioactivity loaded onto the TLC plate.

2.12.4. *Rf* value on TLC

Under the standard conditions described in 2.6.1, the *Rf* is defined as the ratio between the distance moved for a particular compound and the distance moved by the solvent front.

2.12.5. Specific optical rotation

The specific optical rotation ($[\alpha]_D$) was calculated as:

$$[\alpha]_D = \frac{(\alpha' - 100) \times 100}{l.c}$$

where: α' = observed rotation;

l = length of light path in decimetre;

c = sample concentration in g/100ml;

D = the D line of sodium at 589nm.

2.12.6. Specific radioactivity

Specific radioactivity is defined as the number of isotope activity units per unit concentration, i.e. Bq/mol or mCi/mmol etc.

2.12.7. Enzyme activity

Total enzyme activity is defined as the total number of enzyme activity units in a particular sample, i.e., ρKat, Kat or U.

Specific enzyme activity was defined as the number of enzyme units per unit of mass, i.e., ρKat/mg.protein.

2.12.8. Standard deviation of a mean value

Except where indicated otherwise, three replicates were used per experimental treatment and a mean value calculated. The standard deviation (σ) used to express the variation of a mean within the three replicates, was calculated as:

$$\sigma = \sqrt{\frac{\sum \chi^2 - n\bar{\chi}^2}{n}}$$

where: n = number of samples;

$\sum \chi^2$ = sum of squares of samples;

$\bar{\chi}$ = mean value of samples.

Chapter Three:

EXPERIMENTAL RESULTS

3.1. The Nature of Growth and Alkaloid Content of the Suspension Cultures

The mixotrophic cell suspension cultures used throughout this investigation were derived from *Nicotiana tabacum* L cv Wisconsin-38, previously established and optimised in this laboratory by Hobbs in 1987 (see Hobbs, 1989). An essential prerequisite to this study was to determine whether the growth pattern of this culture had altered since establishment. In addition, it was also necessary to re-examine the production of nicotine and nornicotine in this culture, since the major part of this investigation was concerned with feeding either cold or radioactive nicotine as a substrate to cultured cells.

3.1.1. Growth pattern of the suspension cultures

The aim of this experiment was to examine the growth pattern by measuring several parameters, including packed cell volume (PCV), fresh weight and dry weight. To achieve this, 1g fresh weight of cells at the end of a culture cycle was transferred aseptically into each of 24, 250ml conical flasks containing 50ml of fresh standard medium (see 2.2.1) and grown under the conditions described in 2.2.3.1. Cultures were sampled every 3 days, with 3 replicates for each sample, from day 0 up to day 24. At each sampling time, PCV was determined first using the method described in 2.3.1; then the cultured cells were separated from the medium by filtration for fresh weight measurement (see 2.3.2). Finally the dry weight was determined using the fresh weight sample as described in 2.3.3. The results for PCV, fresh weight and dry weight are shown in Figs.3.1. (a), (b) and (c) respectively.

From the results presented, it can be seen that the growth pattern of this culture was very similar for all three parameters. There was a short lag phase of 3 days for fresh

weight (b) and dry weight (c), with a slightly longer lag phase of 6 days for PCV (a). After a long linear-increase phase, the cultures reached a stationary phase around 15-18 days and then remained relatively constant until the end of the 24 day culture period. The increase in biomass over the culture cycle was about 13 fold for PCV, 14 fold for fresh weight and 11 fold for dry weight.

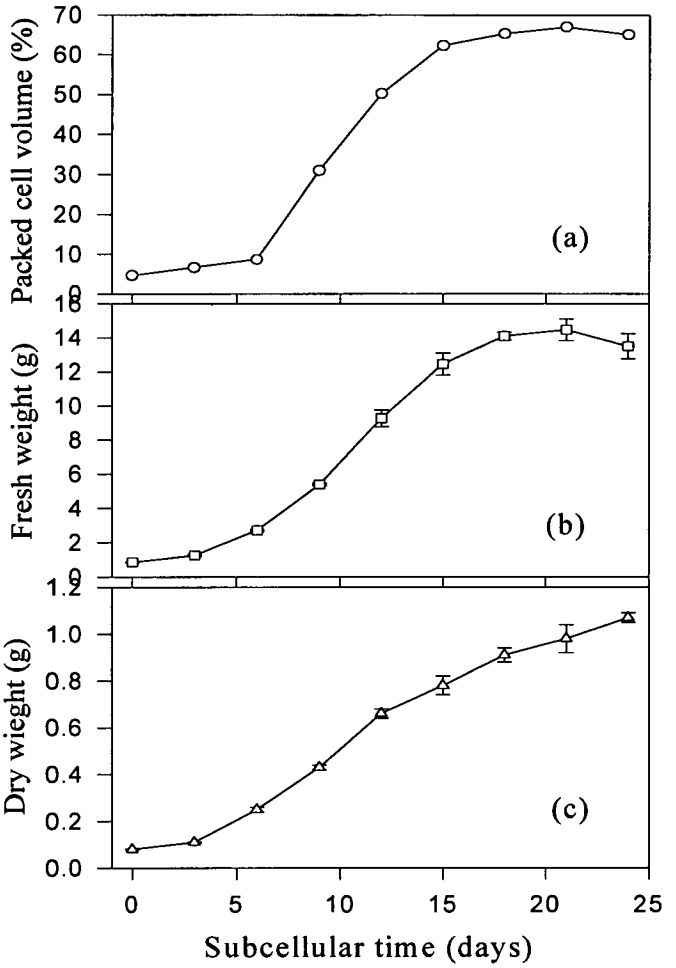


Fig.3.1 Changes in PCV (a), Fresh Weight (b) and Dry Weight (c) During a Culture Cycle of Suspended Cells of *Nicotiana tabacum* L cv. Wisconsin-38

Each value represents the mean of three replicates ± S.D. except PCV (a) which is the mean of three replicates.

It would appear that this suspension culture, previously optimised by Hobbs (1989), responded to fresh medium only 2 or 3 days after inoculation. But, the culture cycle was shorter by about 5 days to that reported by Hobbs & Yeoman (1991b). In his experiments, the onset of the stationary phase was about 5 days later than in this study. This change could have resulted at least in part from the storage of cultures over several years, or the use of a different medium with a lower concentration of growth regulators, or both. It was noted that the suspension cultures became very dense after 15 days in culture.

3.1.2. Production of nicotine and nornicotine by suspended cells over a 25 day culture cycle

It is known that cell suspension cultures of *Nicotiana tabacum* L cv. Wisconsin-38 are capable, not only of accumulating nornicotine as the major alkaloid, but are also able to convert nicotine to nornicotine (Hobbs and Yeoman, 1991a, b). In order to distinguish the alkaloids produced from added nicotine from those produced endogenously, it was necessary to monitor the endogenous content of nicotine and nornicotine throughout a culture cycle. To achieve this, 1g fresh weight of cells at the end of a culture cycle was transferred into each of 15, 250ml conical flasks containing 50ml of fresh medium and grown under the conditions described in 2.2.3.1. The cultures were sampled at 5 day intervals after inoculation, with three replicates for each sample. Alkaloids were extracted using the method described in 2.5.1. The nicotine and nornicotine in the cultures were quantified by HPLC as described in 2.6.2. The results are presented in Fig.3.2.

Fig.3.2. shows that only a small amount of alkaloid is present 5 days after inoculation when the cells are in the early stages of growth, and when only a small amount of

biomass has been accumulated. Nicotine content reached a maximum value early in the growth cycle (around day 10) and subsequently declined to a small but detectable amount at the end of the 25 day culture cycle. In contrast, nornicotine appeared around day 5 of culture and, afterwards, increased considerably to reach a maximum value at day 20, then remained relatively constant until the end of the culture cycle. This suggested that bioconversion activity was initiated earlier than 10 days after inoculation. Nicotine produced around day 5 could have been gradually converted to nornicotine and this bioconversion could have been greater than the production of nicotine around day 10 of culture. Therefore, cultures not older than 10 days were considered to be appropriate for subsequent feeding experiments.

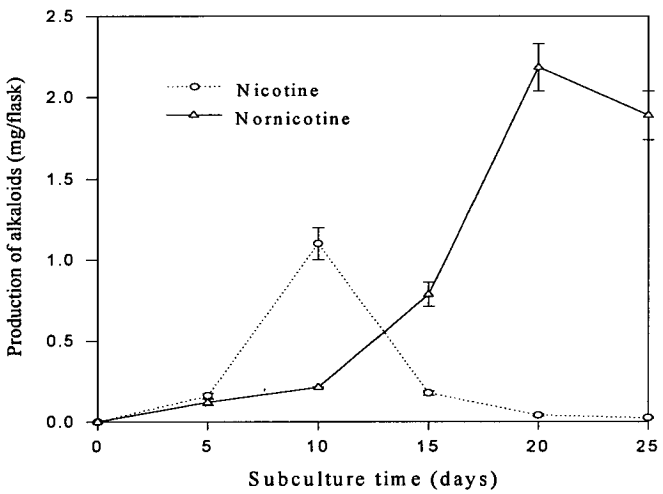


Fig.3.2 Changes in the Endogenous Content of Nicotine and Nornicotine of Cell Suspension Cultures of *Nicotiana tabacum* L cv. Wisconsin-38 over a Culture Cycle.

Each value represents the mean of three replicates ± S.D.

3.1.3. Summary

The results described in 3.1 can be summarised as:

(1) The growth pattern of this cell suspension culture has changed only slightly over the years;

(2) It was confirmed that the cell suspension cultures are still capable not only of accumulating nornicotine as the major alkaloid but also of bioconverting nicotine to nornicotine;

(3) The bioconversion of endogenous nicotine to nornicotine probably occurred before day 5 of culture and gradually increased exceeding the production of nicotine by about day 10. Therefore, cultures not older than 10 days were selected for subsequent feeding experiments.

To ensure that maximum bioconversion rates were achieved, attempts were now made in the next experiment to determine the best time in the culture cycle to add nicotine.

3.2. Bioconversion of Added Nicotine to Nornicotine by Cell Cultures

In the previous experiment (3.1), it was confirmed that the mixotrophic cell suspension cultures are still capable, not only of accumulating nornicotine as the major alkaloid but also, of bioconverting endogenous nicotine to nornicotine. In order to establish the kinetics of this bioconversion, a series of feeding experiments were carried out. But first of all, the efficiency of a number of extraction procedures reported to be effective with the tobacco alkaloids was investigated.

3.2.1. Comparison of several reported procedures for the extraction of nicotine alkaloids

Over the last 40 years, many procedures have been reported for the extraction of nicotine and related pyridine alkaloids, from either tobacco plants or cultures. However, little evidence is available about the efficiency of these extractions. It is very important, especially when dealing with the feeding of compounds to cultured cells, to ensure the reliability of the selected procedures so that accurate experimental data can be produced. A reliable extraction method must give reasonably high recovery rates (extraction efficiency) both for the substrate and its products. In order to select a reliable method, five reported extraction procedures, with some modifications as required, were compared for their extraction efficiency of nicotine and nornicotine.

A mixture of 10mg of nicotine (Sigma) and nornicotine (Rothmans) was added to a 250ml conical flask containing approximately 50ml of a 10 day old tobacco culture. After thoroughly mixing by shaking, the nicotine and nornicotine in the cultures were then extracted immediately using the methods described below. Controls were made without the addition of either alkaloid. After subtraction of the endogenous alkaloids (the controls) from total alkaloids (all determinations by HPLC as described in 2.6.2), the difference values were used for the comparison. Three replicates were used in all these tests.

Method 1. (Hobbs, 1989): the cultures (cells plus medium) were ground up using a pestle and mortar with 35% (*v/v*) ammonium hydroxide to give a final concentration of 2% (*v/v*). To this homogenate an equal volume of chloroform was added and thoroughly mixed. The mixture was left to stand for 5min before the cell debris was removed by filtration under pressure through Miracloth (Calbiochem). The two phases of the filtrate were separated, using a separating funnel, and the aqueous phase re-extracted with a further two volumes of chloroform. The chloroform extract was then evaporated to dryness at 30°C using a Büchi Rotavapor R-110 system. The residue was re-suspended in 10ml of 0.3% (*v/v*) sulphuric acid (BDH) and placed on

ice for 30 to 60min. The sample was then transferred to a separating funnel and the pH adjusted to 10.8 with 35% (v/v) ammonium hydroxide (BDH) followed by extraction with 3×15ml aliquots of chloroform. Dissolved water was removed from the chloroform extract by the addition of 0.5 to 1.0g of anhydrous sodium sulphate (BDH), which was subsequently removed by filtration through a glass microfibre filter (Whatman) under reduced pressure using a Millipore filtration unit (Millipore). The sample was then filtered through a 0.45μm nylon filter (Whatman), supported in a Swinnex disc filter holder unit (Millipore), and transferred to a 1.5ml brown HPLC sample vial prior to HPLC analysis.

Method 2. (Saunders & Blume, 1981): The cultures (cells plus medium) were ground up in 30ml of methanol (BDH) and N HCl in a ratio of 2:3 (*v/v*) with a pestle and mortar. The homogenate was then centrifuged at 500g for 3min and filtered through Miracloth (Calbiochem) in a Büchner funnel. The solution was filtered through a 0.45μm nylon filter (Whatman), supported in a Swinnex disc filter holder unit (Millipore), and transferred to a 1.5ml brown HPLC sample vial prior to HPLC analysis.

Method 3. (Manceau, 1989): The cultures (cells plus medium) were ground up in 30ml of methanol and ammonium hydroxide (35%, *v/v*) in a ratio of 9:1 (*v/v*) with a pestle and mortar and stirred for 3hr. The cell debris was then removed by filtration under pressure and the filtrate was evaporated to dryness at 30°C using a Büchi Rotavapor R-110 system. The residue was treated with 3×10ml of 0.1M Sulphuric acid (BDH) and the acidic aqueous phases were collected. After filtration through a Whatman No.1 filter paper, the pH of the filtrate was adjusted to 9.0 with 35% ammonium hydroxide and the solution then extracted with 3×15ml aliquots of chloroform. Thereafter, the resulting chloroform extract was treated in the same way as in Method 1. (see above).

Method 4. (a modified method of Horwitz, 1970): The cultures were frozen at -70°C and freeze dried (LSL Secfoid) for 7 days. The residue was ground up in 10ml of 20% (*v/v*) ammonium hydroxide in a pestle and mortar and extracted with 3×15ml aliquots of chloroform. Dissolved water was removed from the chloroform extract by the addition of 0.5 to 1.0g of anhydrous sodium sulphate (BDH), which was subsequently removed by filtration through a glass microfibre filter (Whatman) under reduced pressure using a Millipore filtration unit (Millipore). The filtrate was then filtered through a 0.45μm nylon filter (Whatman), supported in a Swinnex disc filter holder unit (Millipore), and transferred to a 1.5ml brown HPLC sample vial prior to HPLC analysis.

Method 5. (Lockwood & Essa, 1984): Potassium hydroxide pellets (BDH) were added to the medium to give a final concentration of 2M. After hydrolysis for 1hr at 90°C, the medium was extracted three times with equal volumes of chloroform. After removing the dissolved water with anhydrous sodium sulphate, the chloroform extract was filtered, through a glass microfibre filter (Whatman), using a Büchner funnel and evaporated to dryness under vacuum at 30°C in a Büchi Rotavapor R-110 system. The residue was then dissolved in 1ml of HPLC grade methanol and then filtered through a 0.45μm filter (Whatman), supported in a Swinnex disc filter holder unit (Millipore). The filtrate was stored in a 1.5ml brown HPLC sample vial prior to measurement.

It can be seen from Table 3.1. that three of the five methods tested gave acceptable results when the two alkaloids were measured by HPLC (HPLC performance). The extracts obtained from Method 2. and Method 3. were extremely unstable making quantification by HPLC impossible. Since Method 3. was used for GC determinations by Manceau et al (1989), it is presumed that it might not be suitable for HPLC analysis. Using Hobbs' method (Method 1.), the best HPLC performance of the five methods tested was achieved, however the percentage recovery, in particular for nornicotine, was very low and the recoveries for both of the alkaloids varied greatly

from replicate to replicate. This may be because the method developed by Hobbs (1989) was a compromise procedure to enable the extraction of four alkaloids with one procedure: nicotine, nornicotine, anatabine, anabasine. The same problems were encountered with the samples obtained using freeze dried material (Method 4.) and here the HPLC peak shapes for both alkaloids were poor (figure not shown). The highest percentage recoveries of 81.20% for nicotine and 69.88% for nornicotine were obtained using Method 5. Here the HPLC performance was the same as that for Method 1. but the recovery variations were much smaller.

Table 3.1. Comparison of five reported methods for the extraction of nicotine and nornicotine

Method	Alkaloids	Recovery (%)*	HPLC performance**	Recovery variation (%)***
1	nicotine	65.46	+++	13.71
	nornicotine	30.38	+++	19.02
2	nicotine	ND	- -	N/A
	nornicotine	ND	- -	N/A
3	nicotine	ND	-	N/A
	nornicotine	ND	-	N/A
4	nicotine	58.85	++	4.61
	nornicotine	26.69	++	16.00
5	nicotine	81.20	+++	4.00
	nornicotine	69.88	+++	0.25

** Recovery represents the percentage of the alkaloids extracted over those added; Values are the mean of three replicates; ND = not detectable.*

*** HPLC Performance indicates the results of HPLC measurements corresponding to a good separation and peak shape of the two alkaloids; +++ = the best and - - = the worst.*

**** Recovery Variation represents the difference between the best and the worst Recovery and values are the mean of three replicates.*

These results as expected show that nicotine and nornicotine behave differently during extraction, although they have a similar chemical structure. The distribution of the

two alkaloids between aqueous phase and organic phase depends on the alkalinity of the extraction medium. Strong alkaliis, such as potassium hydroxide, can cause deprotonation of the *N* atom of the alkaloids aiding distribution towards the organic phase. In addition, some of the contaminating substances present in the extracts may be hydrolysed by the alkali.

Unfortunately, none of the five methods tested gives a recovery rate close to 100%, which suggests that nicotine may be present in a bound form (see Manceau et al 1989). In order to explore this possibility, a mixture of 10mg of nicotine (Sigma) and nornicotine (Rothmans) was added to a 250ml conical flask containing either 50ml of distilled water, 50ml of fresh medium (after sterilisation by autoclaving) or 50ml of 10 day old medium (prepared from a 10 day old cell suspension cultures by separation of cells from medium by filtration under pressure). After mixing up by shaking, the alkaloids in the three treatments were extracted using method 1. (Hobbs, 1989). Three replicates were used in all three test groups.

Table 3.2. shows no marked differences in the percentage recovery of nicotine and nornicotine extracted from the three 'media', although the recoveries of the two alkaloids vary from replicate to replicate among the three media tested. These results indicate that substances in the medium did not seriously affect extraction of the alkaloids. However, the recovery from the extracts of fresh medium is better than that from 10 day old medium (the best was observed from distilled water). From this, it is presumed that some cellular metabolites present in the 10 day old medium affect extraction of the alkaloids. In view of the results presented in Table 3.1., which show that hydrolysis with strong alkali reduces variation between replicates but does not significantly improve extraction of both alkaloids if taking into account for the variations, it would seem that strong bound forms of nicotine are unlikely to exist. (radioactive experiments in later section will confirm this suggestion).

Table 3.2. A Comparison of the Efficiency of Nicotine and Nornicotine Extraction from Different 'Media'

Medium	Alkaloids	Recovery (%)*	Recovery variation (%)**
distilled water	nicotine	77.10	9.25
	nornicotine	67.98	8.37
fresh medium	nicotine	83.00	5.62
	nornicotine	67.96	22.35
10 day old medium	nicotine	70.35	19.95
	nornicotine	49.54	17.11

** Recovery represents the percentage of alkaloids extracted over those added;*
*** Recovery Variation represents the difference between the highest and the lowest Recovery.*
**** These values were obtained after taking into account the endogenous level of alkaloids. Each value is the mean of three replicates.*

The results obtained show that Method 5. is the most suitable for extraction and HPLC analysis of samples containing nicotine and nornicotine. Using this method (with slight modification as described in 2.5.1.), 85.3% of nicotine and 73.6% of nornicotine were extracted from the medium and 79.8% of nicotine and 63.1% of nornicotine from the cells. These values were subsequently used as correction factors in studies on the bioconversion of added nicotine to nornicotine.

3.2.2. Determination of the optimum point in the culture cycle for the bioconversion of nicotine to nornicotine

In previous studies, in this laboratory, on the bioconversion of added nicotine to nornicotine, day 10 of the culture cycle was selected as a suitable feeding point (Hobbs & Yeoman, 1991a). However, the changes to the timing of the culture cycle of this cell line reported earlier necessitated a re-evaluation of the optimum point in the

culture cycle for this bioconversion. Accordingly, 10mg of nicotine (Sigma) was added aseptically to each 250ml conical flask containing approximately 50ml of suspended cells at different points in a culture cycle, i.e. day 0, day 5, day 10, day 15, day 20 and day 25. After the addition of nicotine, all these culture were incubated under standard culture conditions for 24hr when the alkaloids in both medium and cells were extracted using the method described in 2.5.1. Nornicotine in each of the flasks was then quantified by HPLC (2.6.2.). All values were corrected for endogenous amounts of nornicotine.

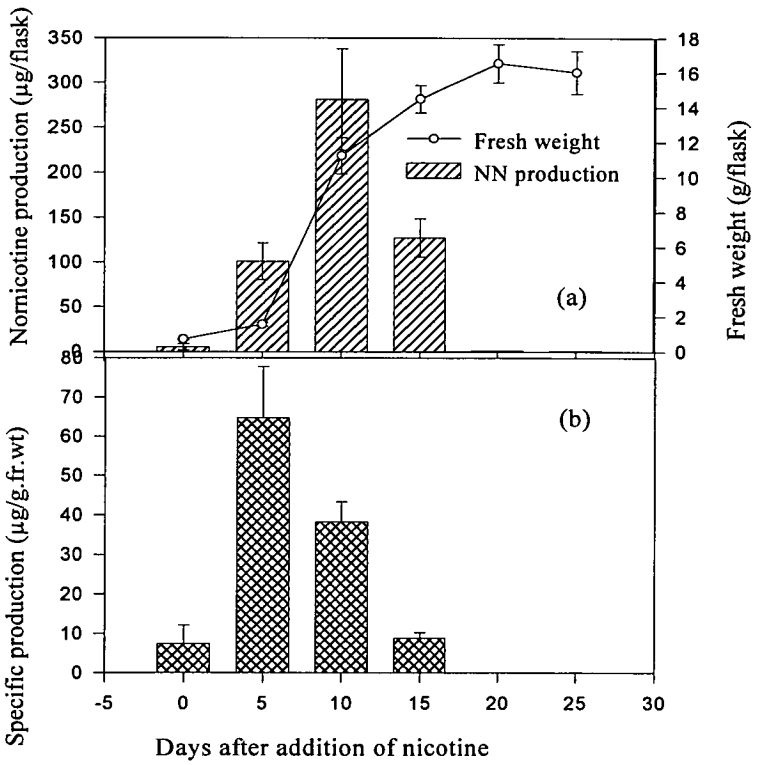


Fig.3.3 Effect of Time of Addition of Nicotine on Nornicotine Production During One Culture Cycle

Flasks were harvested 24hr after the addition of nicotine. Each value represents the mean of three replicates ± S.D.. (a) Culture growth and nornicotine production; (b) Specific nornicotine production.

It can be seen from Fig.3.3b that the maximum amount of nornicotine produced per g.fr.wt. occurred with day 5 cultures after 24hr incubation with nicotine, although a substantial conversion was observed with day 10 cultures. However, only about 10% of the maximum bioconversion capacity appears with day 0 and day 15 cells respectively. No bioconversion occurred with day 20 cells and subsequently.

The results shown in Figs.3.3(a,b) suggest that the maximum bioconversion activity was between day 5 and day 10, although the maximum amount of nornicotine produced per culture appeared at day 10 (see Fig.3.3a). However, the maximum specific nornicotine production occured at day 5 in the culture cycle. As there was only a limited amount of biomass available at day 5, day 10 of the culture cycle was selected as the optimum feeding point.

3.2.3. Can culture medium convert added nicotine to nornicotine?

Results from the previous experiment showed that the optimum feeding point for nicotine was at day 10 of the culture cycle. While it seems likely that the presence of cells is necessary for this bioconversion. It is possible that enzyme(s) released from the cells could be responsible for some or all of the bioconversion activity.

Accordingly, an experiment was performed to determine whether nicotine could be converted to nornicotine by medium alone. Cell cultures at day 5, 10 and 15 were selected to carry out this experiment. After aseptic separation of cells from medium by filtration under pressure, 10mg of nicotine was added to each 250ml conical flask containing 30ml of medium. Control flasks contained 30ml of the same medium as the treatments but without the addition of nicotine. After incubation under standard conditions for 10 days, the alkaloids in both treatments and controls were extracted and quantified by HPLC. The results are presented in Table 3.3.

Table 3.3. Bioconversion of Added Nicotine by Culture Medium

Age of medium	Control (mg/flask)		Treatment (mg/flask)	
	Nicotine	Nornicotine	Nicotine	Nornicotine
Day 5	0.03±0.003	0.03±0.004	10.04±1.11	0.00
Day 10	1.13±0.24	0.25±0.005	9.89±0.98	0.00
Day 15	0.42±0.04	0.78±0.19	8.99±2.01	0.00

Each value represents the mean of three replicates ± S.D. The alkaloids in treatments are corrected for their respective endogenous amounts and the extraction efficiencies.

From the results presented in Table 3.3, it can be seen that none of the three media tested are capable of converting added nicotine to nornicotine, after taking into account for their endogenous amounts and correcting for the extraction efficiencies. All 10mg nicotine added to the different age media were recovered completely after an incubation period of 10 days. It would seem that the enzyme(s) involved are either not released into the medium or inactive. Therefore, the bioconversion activity is associated with the intact cells.

3.2.4. Effect of the concentration of nicotine added to day 10 culture on the bioconversion

The aim of this experiment was to determine the effect of nicotine concentration on the growth of cultures and the bioconversion of added nicotine to nornicotine. 10 day old cell cultures were used in this experiment.

A range of amount of nicotine from 5mg to 160mg were added aseptically to each 250ml conical flask containing 50ml of a 10 day old culture. The flasks were incubated under standard culture conditions for 15 days and, then, the alkaloids in both cells and medium were extracted (see 2.5.1) and quantified by HPLC (see 2.6.2). The results are presented in Fig.3.4.

Fig.3.4 shows that as the concentration of nicotine increases, the amount of nornicotine produced also increases, but this increase is not proportional to the amount of added nicotine. From 10mg to 40mg of nicotine, there is no real increase in the amount of nornicotine produced by the cultures. Subsequently the amount of nornicotine produced remained more or less constant. This suggests that the biconversion activity [enzyme(s)] becomes saturated at about 10mg of nicotine (62.5μmol), the amount used for the further studies. No toxic effects of added nicotine on the appearance of cultures were observed.

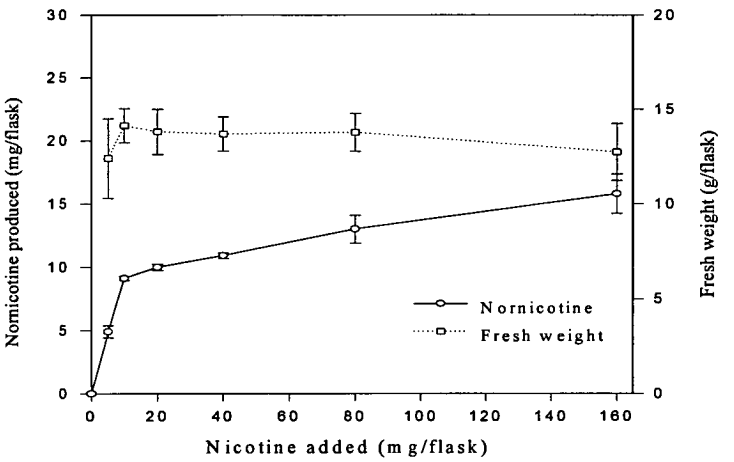


Fig.3.4 Effect of the Amount of Nicotine Added to 10 Day Old Cultures on Growth and Gornicotine Production

Flasks were sampled after 15 days incubation. Each value represents the mean of three replicates ± S.D..

In the next experiment, the kinetics of the bioconversion of added nicotine to nornicotine were studied in some detail.

3.2.5. Kinetics of nicotine bioconversion to nornicotine

In the previous experiments, it was shown that the biconversion activity was associated with intact cells and that an optimum feeding point for nicotine was day 10 of the culture cycle. Having established the basic conditions for the biconversion of added nicotine to nornicotine, it was now decided to determine the kinetics of this bioconversion.

10mg (62.5 μmol) of nicotine were added aseptically to each 250ml flask containing 50ml of a 10 day old cell culture and, these flasks were incubated under standard culture conditions. The flasks were subsequently sampled, in replicates of three at each sampling time, from 5 hours up to 15 days after the addition of nicotine. After separation of the cells from medium by filtration under pressure, the cells were washed three times with fresh medium. The filtrate and the washed medium were then combined and the alkaloids in both cells and medium were extracted (2.5.1) and quantified by HPLC (2.6.2). Having corrected for endogenous levels of nornicotine, the nornicotine produced throughout the incubation course, expressed as a percentage bioconversion (see 2.10.3), is shown in Fig.3.5.

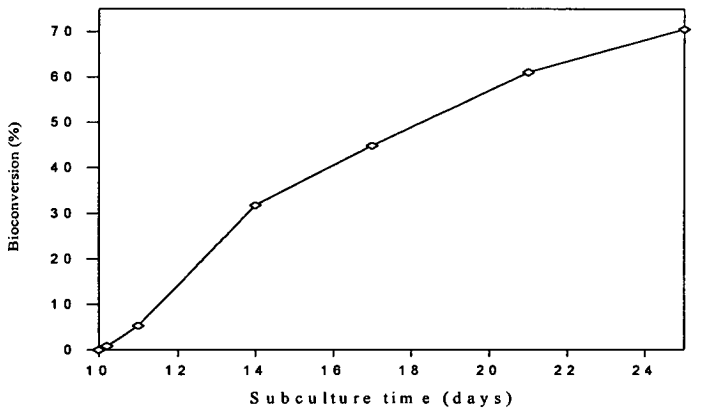


Fig.3.5 Kinetics of the Bioconversion of 10 mg of Nicotine Added to 10 day old Cultures

Values were corrected for the endogenous amounts of nornicotine. Each point represents the mean of three replicates.

It can be seen from Fig.3.5 that only a very small amount of the nicotine added was converted to nornicotine after 5h. Thereafter, the percentage biconversion increased sharply until approximately 70% of the added nicotine had been converted into nornicotine after 15 days incubation. No toxic effects of the added nicotine on the cells were observed. This is consistent with the results shown in Fig.3.4.

A detailed breakdown of the amount of nicotine and nornicotine present in cells and medium is shown in Figs.3.6a and b. It can be seen from Fig.3.6a that the nicotine content of cells increases slightly during the first 5 h accompanying a decrease in the medium. This shows that the added nicotine has been taken up by the cells. Indeed, during the first day, about 4mg out of the 10mg nicotine added has disappeared while, only less than 1mg of nornicotine has appeared. A similar discrepancy was observed by Manceau et al (1989), with a cell suspension cultures of *Nicotiana plumbaginifolia*, who presumed that the nicotine was binding to cellular material. However, binding of nicotine to cell components is unlikely as suggested in 3.2.1 and the radioactive experiments reported later. Such a discrepancy could be explained by the presence of an unknown intermediate(s) involved in this conversion. Some of the discrepancy may also be due to experimental error, because all the values have been adjusted to allow for extraction efficiency. In parallel with the decrease in nicotine content in both cells and medium, there is an increase in nornicotine which increases gradually until a maximum value is reached after 15 days incubation (see Fig.3.6b). Some nicotine is also present in both cells and medium after 15 days.

The results obtained suggest that the biconversion appears to be intracellular, with nicotine being taken up from the medium, and converted to nornicotine which is subsequently released into the medium. After a 15 day incubation, 70% of the total nicotine (10mg) added was converted to nornicotine. This percentage bioconversion by the mixotrophic cell line used is close to that reported by Hobbs and Yeoman (1991a), who achieved a 66% conversion of added nicotine. It would appear that

10mg of nicotine has saturated the bioconversion activity [enzyme(s)] of these cultures. This is consistent with the results presented in Fig.3.4.

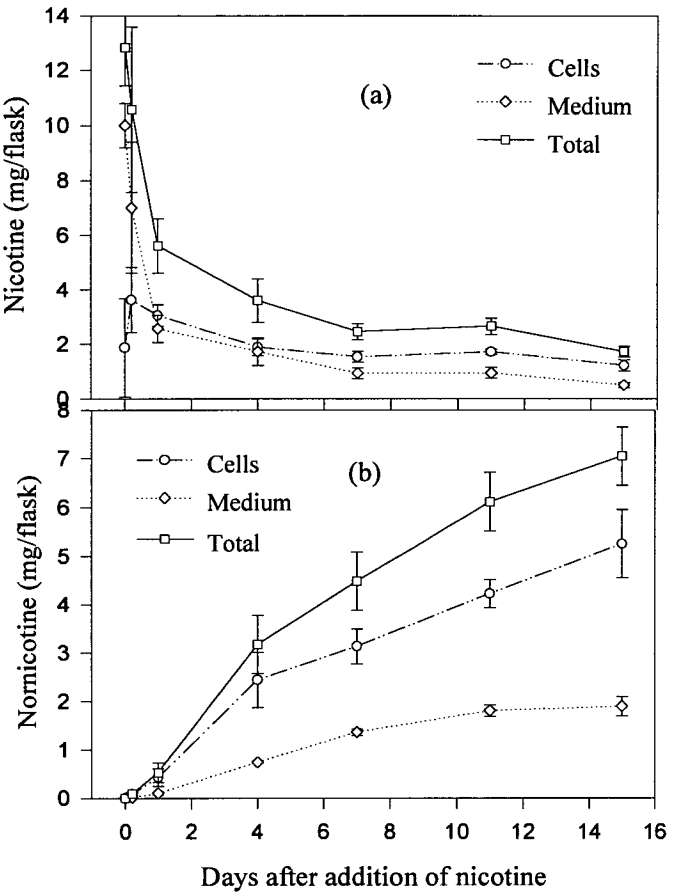


Fig.3.6 Changes in Nicotine (a) and Nornicotine (b) Content of Cells and Medium with Time after Addition of 10mg of Nicotine to 10 Day Old Cultures

Values were corrected for the endogenous amount of both alkaloids. Each point represents the mean ± S.D. of three replicates.

In the next experiment, the effect of biomass of a 10 day old culture on the extent of bioconversion was studied.

3.2.6. Effect of biomass on the extent bioconversion by 10 day old cultures

In the previous experiment, it was shown that about 70% of the added nicotine (10mg) was converted to nornicotine by 10 day old cell cultures in 15 days. The aim of this experiment was to determine whether the amount of biomass of a 10 day old culture affected the bioconversion.

An appropriate amount of 10 day old cell cultures were thoroughly mixed and divided into 3g, 6g and 9g (fresh weight) aliquots resulting in a volume of about 50ml in 250ml conical flasks (54 flasks in total). To 27 out of the 54 flasks (the other 27 flasks were controls), 10mg of nicotine was added aseptically. The flasks, along with the control flasks without added nicotine, were then incubated with shaking under the standard culture conditions. The cultures in each fresh weight group were sampled 5 days, 10 days and 15 days after the addition of nicotine (three replicates for each group at each sampling point) and, subsequently, the alkaloids were extracted (see 2.5.1) and quantified by HPLC (see 2.6.2). The endogenous levels of alkaloids were taken into account by subtracting the control values from the treatment values.

The results presented in Fig.3.7 show a positive correlation between biomass and nornicotine converted from added nicotine throughout the experiment, but, a linear relationship. At the end of the incubation period, approximately 50% of the added nicotine had been converted to nornicotine in the 3g group, whilst more than 70% was converted in the 6g group, which corresponds to the results shown in Fig.3.4. In the 9g group, almost all of the nicotine added (10mg) had been converted into nornicotine after 15 days. This coincides the assumption made by the experiments of 3.2.4 and 3.2.5 that 10mg of nicotine added saturated the bioconversion activity, whilst increasing the biomass of 10 day old cultures improved the extent of bioconversion up to 100%.

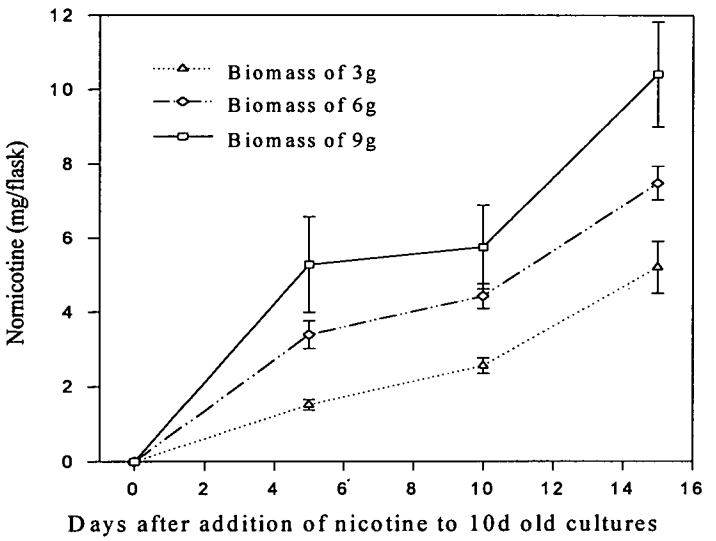


Fig.3.7 Effect of Biomass (10 day old cultures) on the Extent of Bioconversion of Nicotine to Nornicotine

Values were corrected for the endogenous amount of nornicotine. Each value represents the mean of three replicates ± S.D..

3.2.7. Summary

The following points arising from the experiments presented in this section are now summarised:

(1) A reliable extraction procedure has been established for nicotine and nornicotine;

(2) The 10 day old cells of a 25 day culture cycle were selected for further experiments. This represents a compromise between amount of biomass and bioconversion capacity;

(3) The culture medium from 10 day cell cultures was not capable of converting added nicotine to nornicotine;

(4) Increasing the amount of added nicotine to 10 day old cultures increased the amount of nornicotine converted but not proportionally;
(5) It appeared that this bioconversion was intracellular, with nicotine being taken up from the medium, converted to nornicotine and then released into the medium. After 15 days, about 70% of the nicotine (10mg) added was bioconverted to nornicotine;
(6) A 100% bioconversion of the added 10mg nicotine was achieved with 9g of a 10 day old culture.

In order to begin to understand the mechanism of this reaction, attempts were made in the next section to determine the optical properties of the nornicotine converted from added nicotine.

3.3. Determination of the Optical Properties of Nornicotine Produced from Added Nicotine by 10 Day Cultures

In 1961, Kisaki and Tamaki (1961a) detached some leaves from the shoots of *N. tabacum* L. var. *Bright Yellow*, which were then grafted onto a tomato stock, and fed with a 5mM aqueous solution of *l*-(-)-nicotine. After about 100hr, the nornicotine produced from optically pure (-)-nicotine was partially racemized. The presence of partially racemized nornicotine in these extracts of tobacco leaves led to the formulation of a hypothesis (see Fig.1.2) by Leete and Chedekel (1974). In this hypothesis, it was proposed that the bioconversion was stereospecific and that racemized nornicotine was formed as a result of the opening of the pyrrolidine ring during *N*-demethylation. Since then, no real attempt has been made to validate this hypothesis. The biconversion system, previously developed by Hobbs and Yeoman (1991a) and refined in this study, provides an opportunity to test this hypothesis at least in cell cultures.

In these experiments, 10mg of (-)-nicotine (Sigma) was added to each of three 50ml 10 day old cultures in 250ml conical flasks. These cultures were harvested after incubation under standard conditions (2.2.3.1) after 10 days and the alkaloids extracted using the method described in 2.5.2. After separation on preparative TLC (2.6.1), nicotine and nornicotine were identified under UV light, scraped off and extracted with 3×15ml aliquots of HPLC methanol. Subsequently, the methanol extracts were separated from the silica by centrifugation at 2,000*g* for 10min. The volume of the samples was then reduced to 1ml by evaporation at 35°C under pressure. The samples were finally filtered through a 0.45μm pore size filter unit (Millipore) prior to analysis by polarimetry and chiral GC.

3.3.1. Polarimetric studies

The optical rotation of the samples and authentic standards of nicotine and nornicotine were determined using a Perkin-Elmer Model-141 polarimeter (589nm) at 20±5°C. A standard 1cm sample cell was used. The specific rotation ($[\alpha]_D$) was calculated as described in 2.12.5.

It can be seen from Table 3.4 that the specific rotation of nornicotine converted from added (-)-nicotine by the cell cultures is very close to that of pure (-)-nornicotine, suggesting that no (+)-nornicotine was formed from (-)-nicotine; in other words, no racemization had occurred. It was also observed that the added (-)-nicotine which remained had not changed optically as a result of the incubation.

In order to further confirm the reliability of this result, the same sample of nornicotine converted from added (-)-nicotine was sent to Rothmans International Services Ltd, Bremen for chiral GC determination.

Table 3.4. The Optical Rotations of Nicotine and Nornicotine

	$[\alpha]_D$		
	(-)-form	(±)-form	Sample
Nornicotine*	-40.5° (c=7.5)	-26.0° (c=7.5)	-42.0°
Nicotine**	-134.0° (c=8.2)	0.0° (c=10.0)	-132.0° (c=9.8)

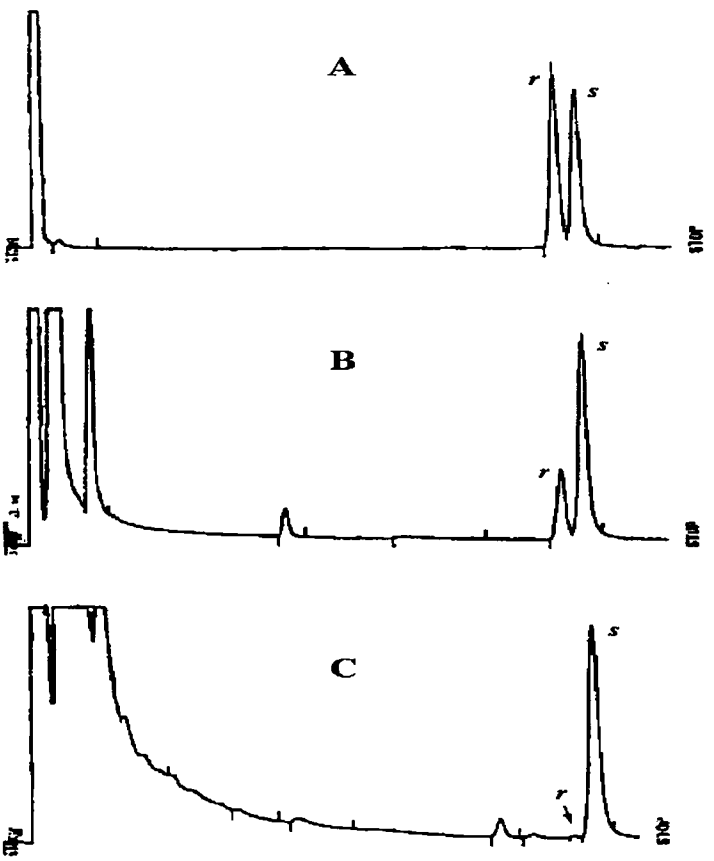
** (-)-nornicotine (purity>98.5%) and (±)-nornicotine (natural nornicotine, purity>91.5 %) were standards provided by Rothmans International Services Ltd.; the sample is the nornicotine converted from added (-)-nicotine by the cell cultures.*
*** (-)- and (±)-nicotine are standards from Sigma; the sample is the nicotine left after bioconversion at the end of a 10 day incubation.*
Each value represents the mean of three replicates of each sample.

3.3.2. Studies using chiral gas chromatography

After the measurement of polarimetry, 6mg of the same nornicotine sample, converted from added nicotine by the 10 day cell cultures, was sent to Rothmans International Services Ltd., Bremen. The optical property measurement of the nornicotine sample was performed by Dr. Elmenhorst using chiral GC. Before the measurement, nornicotine in the sample was derivatised using 0.1M (-)-camphanic acid chloride in CH_2Cl_2 to produce the *N'*-camphanoyl-nornicotine diastereoisomeric amide derivatives, which were then separated by capillary GC on a 30m DB5 wide-bore column, with an oven temperature of 260°C with helium as the carrier gas. The details of the whole processes are described in 2.6.3.2.

Three gas chromatograms are shown in Figs.3.8: *A* is the (+)-,(-)-nornicotine racemate (synthetic nornicotine), *B* is natural nornicotine isolated from Burley tobacco and *C* is the nornicotine converted from added (-)-nicotine by the cell cultures. These chromatograms clearly show that the synthetic nornicotine is a racemate which contains 52% (+)- and 48% (-)-nornicotine. The natural nornicotine contains 25% (+)-

and 75% (-)-nornicotine. The nornicotine obtained by bioconversion of added nicotine by cell cultures shows (+)-nornicotine only as a minute trace. This result is firmly consistent with that obtained from polarimetric analysis in 3.3.1 suggesting that the nornicotine obtained by bioconversion of added nicotine contains only one enantiomer exclusively. This provides strong evidence that the *N*-demethylation of nicotine by tobacco cell cultures involved no opening of the pyrrolidine ring as hypothesised by Leete & Chedekel (1974) in tobacco plant.



Figs.3.8 Gas Chromatograms of Chiral Analysis of Nornicotine

N-camphanoyl-derivates of A: (±)-nornicotine (synthetic), B: nornicotine from Burley tobacco and C: nornicotine derived from added (-)-nicotine by tobacco cell cultures

3.3.3. Summary

The following points arising from the results presented in this section are now summarised:

The results obtained from two different techniques, polarimetry and chiral GC, confirm that the nornicotine bioconverted from added (-)-nicotine by the cell cultures contains only one enantiomer with no evidence of racemization during the bioconversion.

In the next section, ^{14}C ring-labelled nicotine was used as the substrate in a series of feeding experiments to determine the fate of ^{14}C-nicotine added to 10 day old cultures and, to discover whether there are any intermediates or byproducts of the bioconversion.

3.4. Studies on Nicotine Bioconversion to Nornicotine with ^{14}C Ring-labelled Nicotine

In Section 3.3, it was shown that the nornicotine converted from added (-)-nicotine by cell cultures remained optically unchanged suggesting that the biconversion does not involve an opening of the pyrolidine ring moiety of the alkaloid molecule. This result is inconsistent with the hypothesis of Leete and Chedekel (1974) for the intact plant. Therefore, in order to elucidate the mechanism of this reaction, further investigations were required. Towards this end, ^{14}C ring labelled nicotine (*NEN*, Du Pont) was employed to explore the early stages in the conversion of nicotine to nornicotine including the identification of any possible intermediate(s). But first of all, attempts were made to determine the possibility of nicotine binding to cell components.

3.4.1. Is added nicotine bound to cell components during feeding experiments?

It was suggested in 3.2.1 that a bound form of nicotine is unlikely to exist. In order to confirm this assumption, two lines of investigation were pursued in which the distribution of radioactivity in the cultures was studied after the addition of ^{14}C-nicotine and, also, the products of the were separated by gel filtration to identify possible bound forms of ^{14}C-nicotine.

In this experiment, 1.0μCi (1.0μl, containing 2.22×10^6dpm) of ^{14}C-nicotine was added to 10ml aliquots of a randomized mixture of 10 day old cell culture (achieved by pipetting) in each of three 50ml conical flasks. These cultures were incubated under standard culture conditions for 24hr and, then, the cells were separated from the medium by filtration under reduced pressure. Alkaloids in both cells and medium were extracted using the method described in 2.5.1 and the radioactivity in all fractions, including the cell debris, were determined by scintillation counting.

Table 3.5. Distribution of Radioactivity in the Cultures 24hr after the Addition of ^{14}C-Nicotine

Fractions		Radioactivity (dpm×10^5)	Recovery (%)*
Chloroform	Medium	6.2±0.2	27.9
	Cells	10.4±0.4	46.9
Aqueous	Medium	1.7±0.1	7.7
	Cells	1.5±0.1	6.8
Cell debris		0.6±0.1	2.7
Total recovered		20.4±0.9	92.0

** Recovery (%) is the percentage of radioactivity extracted in each fraction as a proportion of that added;*
*** Aqueous fraction is the aqueous phase of either medium or cells remaining after extraction with $CHCl_3$. Values for radioactivity are means of three replicates ± S.D..*

The results presented in Table 3.5 show that 92% of the added radioactivity can be accounted for following extraction, about 75% was recovered in the $CHCl_3$ phase (cells + medium), 15% present in the aqueous phase (cells + medium) and 2.7% remained in cell debris. Eight percent of the added radioactivity was unaccounted for. This is probably due to some of the radioactivity being absorbed onto the glassware.

These results show that using this method (2.5.1), an extraction efficiency of 75% was achieved. This extraction efficiency was slightly lower than for non-radioactive alkaloids (81.2%±4%, see Table 3.1), probably appearing that the extraction efficiency obtained from radioactive nicotine was more accurate than that from non-radioactive one. It was also observed that only 2.7% of the ^{14}C-nicotine added was associated with the cell debris, suggesting that there may be some slight binding of nicotine to some of the cell components but, the level of the binding is very low in contrast to the results reported by Manceau et al (1989) in which a cell suspension culture of *Nicotiana plumbaginifolia* was used.

However, it can be seen from Table 3.5 that a significant amount of radioactivity (about 15% of added ^{14}C-nicotine) is present in the aqueous phase of either medium or cells. In order to discover whether there were any bound forms of nicotine involved, these aqueous phases were subjected to gel filtration. A Sephadex G-25M column PD-10 (Pharmacia) was selected, as this is effective in the separation of small molecular weight solutes from macromolecules (over 5,000MW). From this, it may be predicted that, if bound forms of nicotine exist, there would be at least two peaks along the elution profile corresponding to the free and bound forms of nicotine.

Prior to applying the sample, the column was equlibrated with 50ml of 0.2M phosphate buffer (pH7.5) under gravitational flow. The flow rate of the outlet was controlled at 5ml/min. The elution was then stopped and 0.5ml of the aqueous mixture

obtained from both medium and cells after orangic extraction (see Table 3.5) was added carefully to the top of the column. Elution was then continued. Meanwhile, thirty 1ml samples of the eluate were collected in 10ml test tubes. The radioactivity in each tube was determined as described in 2.6.4.3. A control was used with 0.01μCi of ^{14}C-nicotine (*NEN*, Du Pont) (prepared in the same buffer as the eluant) and this was treated as the aqueous sample. Three replicates for both treatment and control were used and an elution curve was produced plotting elution volume against radioactivity.

It can be seen from Fig.3.9 that there is only one peak in the aqueous phase and in the control. This indicates that bound forms of nicotine are unlikely to be present in the aqueous phase. It would appear therefore that the distribution of nicotine between the aqueous and organic phase during organic extraction depends on the physico-chemical properties of the molecule, because both nicotine and nornicotine are miscible with water.

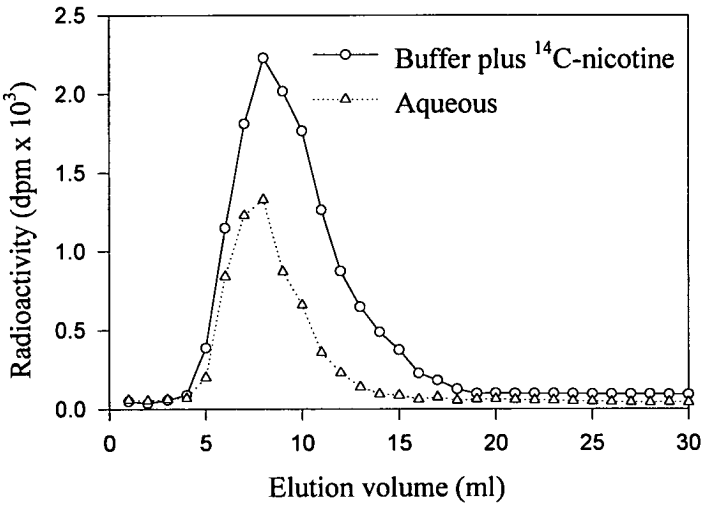


Fig.3.9 The Sephadex G-25 Elution Curve of ^{14}C-Nicotine and Possible Bound Forms of Nicotine

After establishing the effectiveness of ^{14}C-nicotine extraction and failing to find firm evidence of bound forms of nicotine, attempts were now made to study the kinetics of bioconversion of ^{14}C-nicotine bioconversion to nornicotine and to discover whether there were any intermediates or by-products of this bioconversion.

3.4.2. Kinetics of ^{14}C-nicotine bioconversion to nornicotine

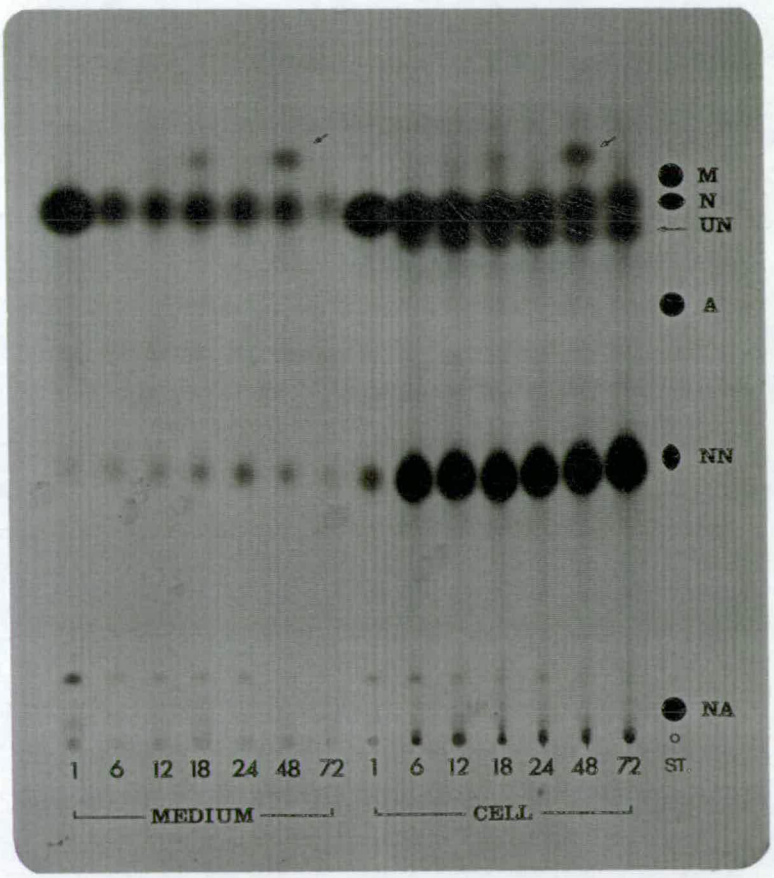


Fig.3.10 Autoradiograph of a TLC Plate Showing a 72hr Time Course of the Bioconversion of ^{14}C-Nicotine to Nornicotine

Numbers beneath the loading points indicate the sampling time in hours after the addition of ^{14}C-nicotine to a 50ml flask containing 10ml of a 10 day old cell culture. N = nicotine, NN = nornicotine, UN = unknown compounds, NA = nicotinic acid, M = myosmine, A = anatabine and St. = a mixed sample of non-radioactive authentic standards which were drawn according to their spots visualised by the method in 2.6.1 after autoradiography.

In 3.2.5, the kinetics of biconversion of added nicotine to nornicotine using 10 day old culture were studied. The results obtained showed that the bioconversion is intracellular and a maximum conversion of 70% was achieved. In order to begin to understand the early-state kinetics of this bioconversion and to establish whether any intermediate(s) were involved, it was necessary to feed ^{14}C-nicotine to 10 day old cultures and sample intensively over a 72hr time course.

10ml aliquots of a randomized mixture of 10 day old cell cultures were tranferred aseptically to 24 conical flasks (50ml), to which 5µl of ^{14}C-nicotine (corresponding to 0.5µCi, 9.5×10^{-3}µmol) was added. All flasks were incubated under the standard culture conditions (see 2.2.3.1). Three flasks were sampled at 1hr, 6hr, 12hr, 18hr, 24hr, 48hr and 72hr and the alkaloids in each flask were extracted (see 2.5.1). Subsequently, the extracted alkaloids were separated one dimensionally by TLC (see 2.6.1). All labelled compounds on the TLC plate were first visualised by autoradiography and then scraped off and counted for radioactivity.

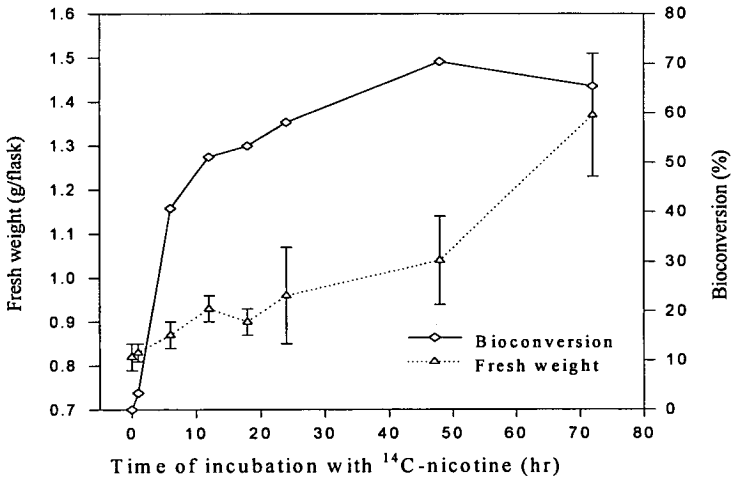


Fig.3.11 Change in Fresh Weight and Kinetics of the Bioconversion of ^{14}C-Nicotine Added at Day 10 of Subculture

Each point represents the mean of three replicates ± S.D. (fresh weight) or the mean value of three replicates.

Fig.3.10 is an autoradiograph of a TLC plate illustrating the bioconversion of ^{14}C-nicotine to nornicotine. It can be seen that nornicotine appears 1hr after the addition of nicotine in both cells and medium and has increased, by 6hr, particularly in the cells. The decrease in radioactivity of nicotine drops sharply in the medium at 6hr with a corresponding increase in the cells. After 6hr the nicotine content in both cells and medium gradually decreases. It can also be seen from Fig.3.10 that several labelled compounds other than nicotine and nornicotine are present during the 72hr incubation, although nornicotine is obviously the predominant product of added nicotine.

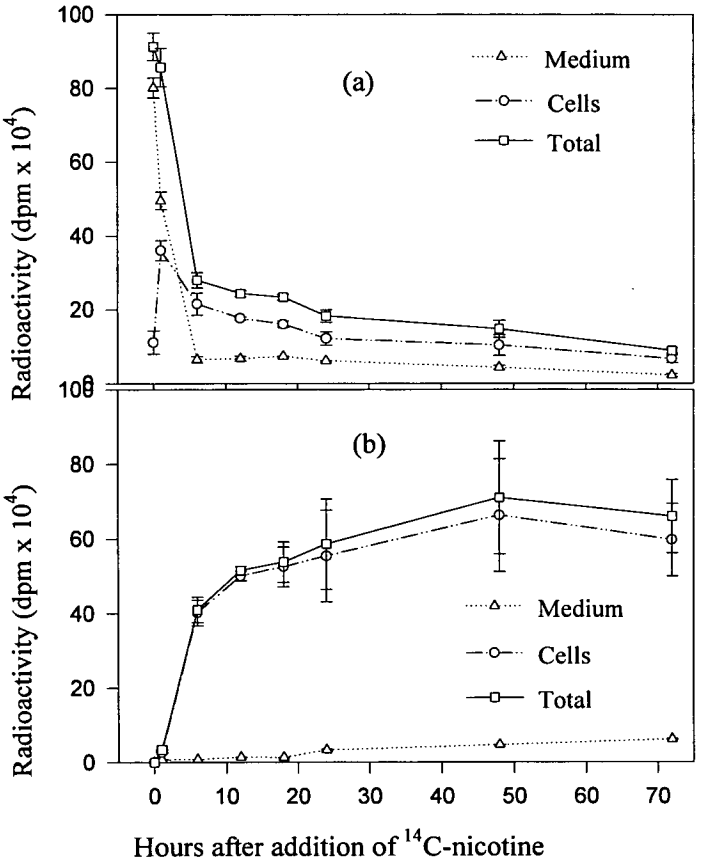


Fig.3.12 Change in the Radioactivity in Nicotine (a) and Nornicotine (b) in Cells and Medium after the Addition of ^{14}C-Nicotine (0.5μCi, 9.5×10^{-3}μmol) at Day 10 of Subculture

Each point represents the mean of three replicates ± S.D..

The kinetics of bioconversion are shown in Fig.3.11 exhibiting that the bioconversion of ^{14}C-nicotine had begun within 1hr and a dramatic increase had taken place over the first 6hr. Subsequently, there was a steady increase in the percentage bioconversion until 48hr when 70.3% of the total recoverable nicotine had been converted. There was only a small increase in fresh weight by the end of the experiment.

Further details of the bioconversion are shown in Figs.3.12ab, from which it can be seen that during the first 6hr more than 80% of the added ^{14}C-nicotine has disappeared from the medium (Fig.3.12a). This loss can be partially accounted for by uptake into the cells, since the radioactivity in the cell fraction increased significantly within the first hour of incubation before decreasing. Also, the disappearance of some of the ^{14}C-nicotine from the medium can be accounted for as bioconverted nornicotine (see Fig.3.12b). In addition, more than 90% of the nornicotine produced appears to be associated with the cell fraction throughout the incubation period. Only a small proportion of the nornicotine produced was released into medium.

3.4.3. Identification of unknown compounds derived from added ^{14}C-nicotine using GC-MS

It can be seen from Fig.3.10 that several labelled compounds other than nicotine and nornicotine appeared in the cell fraction 6hr after the addition of ^{14}C-nicotine and persisted until the end of the experiment. Some of these compounds with a lower R*f* than the unknown compound (UN) are due to impurities in the ^{14}C-nicotine (see Fig.2.3D). In order to establish whether any of these labelled compounds are intermediate(s) of nicotine bioconversion to nornicotine, 10mg of (±)-nicotine (Sigma) were added to 50ml of a 10 day old cell culture in five 250ml conical flasks. These cultures were then incubated under the standard conditions for 72hr After removing cells from medium by filtration, the alkaloids in the cell fraction were

extracted and separated by preparative TLC using the method described in 2.6.5.1. The spots with a higher *Rf* than UN inclusively (see Fig.3.10) were located on the TLC plate by co-elution with a 48hr radioactive sample from the radioactive feeding experiment described in 3.4.2. The area coinciding with those labelled compounds was subsequently scraped off the plate and mixed with HPLC grade methanol. The volume of the methanol phase was then reduced to 1ml under vacuum and finally filtered through a Millipore filter unit with a 0.45μm pore size filter prior to GC-MS analysis.

Pyridine alkaloids are a group of compounds with a similar chemical skeleton, some even with the same molecular weight. However, their mass spectral fragmentation patterns vary from each other therefore making it possible to identify these compounds. Four peaks from the above sample were recorded by GC and their mass spectral fragmentation patterns, together with those of some authentic standards of tobacco alkaloids, are presented in Table 3.6.

It is likely that Peak 2. is nicotine, because it presents the same key diagnostic fragments (*m/z*) and a very similar relative intensity as nicotine, indeed it also shows the corresponding retention time (*R.T.*) to the nicotine standard. Peaks 1. and 3. could not be interpreted using the mass spectra library supplied with the GC-MS system. Peak 1. is unlikely to be nicotine, although it shows a similar fragmentation pattern to nicotine. The fragment of 147 in m/z corresponding to the $(M\text{-}CH_3)^+$ ion, which is a typical molecular ion minus of nicotine MS fragmentation pattern, is absent in Peak 1., but has a different retention time (1.11) from that of nicotine (4.43). In addition, Peak 1. is unlikely to be anabasine, a proposed nicotine metabolite (Waller and Nowacki, 1978). Although they both show a similar parent ion, $161(M\text{-}H)^+$ for Peak.1. and $162(M)^+$ for anabasine, their MS fragmentation patterns are different from each other.

Table 3.6. The Mass Spectral Fragmentation Patterns of Alkaloids Derived from Added (±)-Nicotine and Some Authentic Standards as detected by GC-MS

Alkaloids*	*R.T.* ** (min)	*m/z* (relative intensity [%])***
(±)-Nicotine (st.)	4.43	162(M^+, 57.5), 147(10.9), 133(64.3), 119(49.4), 105(31.5), 92(44.9), 84(100), 78(41.5), 64(44.7), 50(46.8)
(-)-Anabasine (st.)	ND	162(M^+, 100), 145(12.2), 130(10.4), 118(11.7), 105(45.3), 92(6.4), 80(20.9), 63(8.3), 54(43.8), 51(20.9)
(-)-Cotinine (st.)	ND	176(M^+, 23.4), 162(27.7), 156(10.6), 146(14.9), 133(24.5), 118(23.4), 105(7.5), 98(46.8), 92(5.9), 84(100), 78(10.4), 65(5.9), 51(8.7)
(-)-Nornicotine (st.)	5.95	147[$(M-H)^+$, 64.3], 130(11.4), 119(100), 105(38.1), 93(30.7), 80(52.4), 70(94.1), 65(27.1), 51(36.2)
Peak 1.	1.11	161(M^+, 34.5), 133(41), 119(12.9), 105(4.3), 92(8.6), 84(100), 78(5.2), 65(7.8), 51(8.6)
Peak 2.	4.36	162(M^+, 50), 47(8.8), 133(63.6), 119(25), 105(10), 92(20.7), 84(100), 78(14.3), 65(17.9), 51(21.4)
Peak 3.	11.81	159(M^+, 4.3), 111(4.7), 91(12.9), 78(4.3), 71(38.8), 60(21.1), 44(100)
Peak 4.	13.13	176(M^+, 100), 158(48.9), 147(85.1), 130(12.8), 119(77.7), 105(48.9), 98(50.6), 92(38.3), 78(27.5), 70(58.5), 65(29.8), 51(36.2)

** The purity of the authentic standards (st.) is more than 98% for nicotine and 98% for cotinine both from Sigma; 96% for anabasine and more than 98.5% for nornicotine supplied by Rothmans International Services Ltd.*

*** R.T.: the retention time on GC; ND: not detectable.*

**** m/z: the abundance of ions of different mass-to-charge ratios; relative intensity was calculated as a percentage of the peak height of the molecular ion minus over it's parent ion.*

It also can be seen from Table 3.6 that Peak 4. is unlikely to be cotinine, another metabolite of nicotine. Although they both have the same parent ion of 176$(M)^+$, the key diagnostic fragments (m/z) are different from those of cotinine, e.g. 162 ion, a typical fragment of cotinine, is not present in Peak 4.. It can be deduced from the possible chemical structure that this may be a molecule with a formula of $C_{10}H_{12}N_2O$. In its mass spectral fragmentation, 176(M^+,100) represents the parent ion; 158(17.5)

represents $(M-H_2O)^+$ and 147(85.1) represents $(M-CHO)^+$. There is also a fragment of 78(27.5), which is common to all pyridine alkaloids and represents (pyridine moiety)$^+$ or (M -*N*-formylpyrrolidine moiety)$^+$. In addition, the mass spectral fragmentation pattern of Peak 4. is consistent with that of *N*-formyl-3'-nornicotine as described by Braumann et al (1988, restricted communication). Accordingly, it is proposed that the compound represented by Peak 4. as detected by GC-MS is most likely to be *N*-formyl-3'-nornicotine.

3.4.6. Summary

The following points emerged from the results presented in this section are summarised below:

(1) Radioactive experiments confirmed that bound forms of nicotine are unlikely to exist;
(2) The biconversion of ^{14}C-nicotine to nornicotine by 10 day old cell cultures was intracellular showing a maximum conversion of 70.3% by the end of a 72hr incubation.
(3) Several compounds other than nornicotine became labelled during a 72hr incubation with ^{14}C-nicotine. One of these compounds was tentatively identified as *N*-formyl-3'-nornicotine.

In the next section the enzymatic nature of this bioconversion was studied.

3.5. Development of an *in vitro* Assay Procedure for the Enzyme(s) Catalysing the Bioconversion of Nicotine to Nornicotine

The experimental data published on the bioconversion of nicotine to nornicotine over the last decade indicate that this reaction in either plants or cell cultures of tobacco is enzymatically catalysed (Leete & Chedekel, 1974; Poulton, 1981; Wallex & Nowacki, 1978). Recently, the enzyme(s) which catalyses this bioconversion has been demonstrated in the microsomes of the intact plant of *Nicotiana otophora* (Chelearajan, et al, 1993). Whilst, it is well known that tobacco cell cultures are capable of the bioconversion of nicotine to nornicotine (Barz, et al, 1978; Manceau, et al, 1989; Hobbs & Yeoman, 1991a), the enzyme(s) catalysing this process in cultured cells has not been reported. In order to understand the mechanism of this reaction, it is necessary to isolate the enzyme(s) which is responsible for this conversion. The aim of the experiments in this section was to develop an *in vitro* enzyme assay procedure, to enable the nature of the enzyme(s) and the enzymatic mechanism of this bioconversion to be revealed.

To establish an effective *in vitro* enzyme assay, it was first necessary to select the materials to be employed for extraction and assay.

3.5.1. Selection of materials and general considerations

In view of the observation that 10 day old cell suspension cultures of *N. tabacum* L. cv. Wisconsin-38 were able to *N*-demethylate added nicotine to nornicotine with high efficiency, it was decided to use 10 day old cell cultures. The buffer system chosen for the preliminary studies was 0.1M Tris-HCl buffer at pH9.0 supplemented with 3mM DTT and 5mM EDTA. After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with the buffer system at a ratio of 1:1 (cells/buffer, w/v). Following filtration through two layers of muslin, 0.5ml of the resulting cell free extract was then transferred into a 1.5ml Eppendorf centrifuge tube and the assay performed as described in 2.7.3, except that the reaction time was 60min.

In order to minimise any possible effects of metal ions on enzyme activity, distilled (DW) and double-distilled water (DDW) were used to prepare the buffer (Buffer A) as described in 2.7.1. The effects of these two buffers, supplemented with different amounts of DTT and EDTA, on enzyme activity (represented as ^{14}C-nornicotine production) were examined. Three replicates were employed for each treatment along with a boiled control.

Table 3.7. Comparison of Distilled Water(DW) and Double-distilled Water (DDW) in Tris-HCl buffer Containing Different Amounts of DTT and EDTA on Enzyme Activity

Buffer A (pH9.0) plus	^{14}C-nornicotine production in 60min (μmol×10^{-5}/tube)
Control	1.21 ± 0.57
DW+3mM DTT+5mM EDTA	3.80 ± 0.27
DDW+3mM DTT+5mM EDTA	7.14 ± 0.80
DDW+6mM DTT+5mM EDTA	1.89 ± 0.31
DDW+3mM DTT+10mM EDTA	2.23 ± 0.29

** Control was a boiled cell free extract made up with Buffer A (DDW) supplemented with 3mM DTT and 5mM EDTA.*
*** Buffer A (DW) was prepared with distilled water and Buffer A (DDW) prepared with double distilled water.*
All values, minus the control, are the mean ± S.D. of three replicates.

It can be seen from the results presented in Table 3.7 that Buffer A (DDW) supplemented with 3mM DTT and 5mM EDTA is more effective than Buffer A (DW) with the same supplements in the enzyme assay. It is generally believed that there are fewer ions in double-distilled water than in single distilled water and, that the ions in the later may denature the enzyme during disruption of the cells. It was also observed that the highest ^{14}C-nornicotine production in the test was obtained

with Buffer A (DDW) supplemented with 3mM DTT and 5mM EDTA, whilst increasing the amount of either DTT or EDTA decreased ^{14}C-nornicotine production. Therefore, a buffer system contained 3mM DTT and 5mM EDTA prepared with double-distilled water was used in all future experiments. Next, an optimum buffer system was developed for this enzyme assay.

3.5.2. Selection of the buffer system

In order to find an appropriate buffer system for this assay, three buffers supplemented with 3mM DTT and 5mM EDTA were investigated. They were Buffer A [0.1M Tris-HCl buffer (pH9.0)], Buffer B [0.1M potassium phosphate buffer (pH7.5)] and Buffer C [0.1M Glycine-NaOH buffer (pH9.5)]. After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with each of the buffer systems at a ratio of 1:1 (cells/buffer, *w/v*). Following filtration through two layers of muslin, 0.5ml of the resulting cell free extract was then transferred into a 1.5ml Eppendorf centrifuge tube and the assay performed as described in 2.7.3, except that the reaction time was 60min. Three replicates were employed for each treatment with a boiled control. The results are shown in Table 3.8.

Table 3.8. Comparison of Different Buffers on Enzyme Activity

Buffer*	^{14}C-nornicotine production (dpm/tube)
Control	1095 ± 153
Buffer A (pH9.0)	9898 ± 496
Buffer B (pH7.5)	2535 ± 224
Buffer C (pH9.5)	6360 ± 165

** Control was made up with Buffer A and boiled before the assay. All values, after subtracting the control, are the means±S.D. of three replicates.*

It can be seen from Table 3.8. that the highest production of ^{14}C-nornicotine (9898dpm per tube) was achieved with 0.1M Tris-HCl buffer (Buffer A) at pH9.0. Although 0.1M Glycine-NaOH buffer (Buffer C) at pH9.5 was also effective (6360dpm per tube), 0.1M potassium phosphate buffer (Buffer B) at pH7.5 was not suitable (2535dpm per tube).

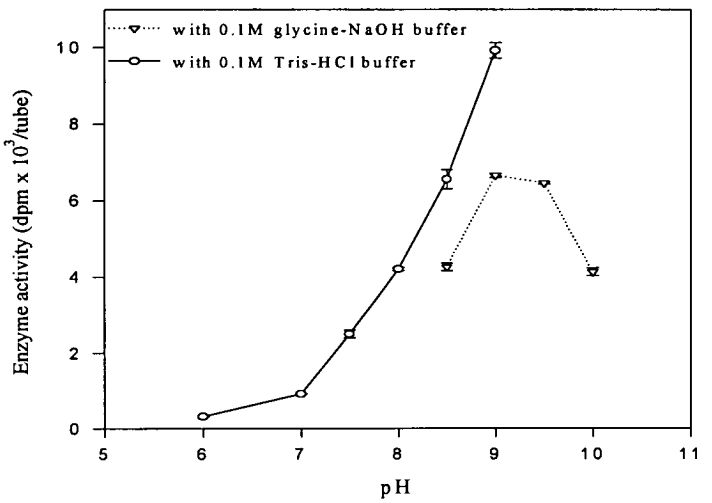


Fig. 3.13 Effect of pH on Enzyme Activity

Each point is the mean of three replicates ± *S.D.*.

In order to determine the optimum pH for this enzyme assay, Buffer A and Buffer C, supplemented with 3mM DTT and 5mM EDTA, were used over a pH range of 6.0 to 10.0 (see 2.7) with a reaction time of 60min. The effect of pH on enzyme activity is shown in Fig.3.13, indicating that the pH optimum for this reaction lies between 9.0 and 9.5. Having established that a buffer system of 0.1M Tris-HCl (pH9.0) supplemented with 3mM DTT and 5mM EDTA gave satisfactory results, it was selected for further development of the enzyme assay.

3.5.3. Protection of the enzyme(s) during cell disruption

Oxidation of sulfhydryl groups and the deleterious effects of heavy metal ions during the preparation of cell-free extracts can be usually prevented by the addition of thiol compounds and EDTA, respectively, to the extraction buffer. However, there are other causes of enzyme loss, for example, those resulting from quinone compounds, medium polarity and ionic strength, inhibition of proteases etc. These must also be considered when preparing cell-free extracts, because these factors can seriously affect the activity of the enzyme. Although EDTA in an appropriate amount can protect the enzyme from some of these factors, an excess amount will result in denaturation of the enzyme (see 3.5.1).

In order to investigate the effect of these factors on enzyme activity, four compounds were added to 0.1M tris-HCl (pH9.0) supplemented with 3mM DTT and 5mM EDTA. They were 0.25M of sucrose and 1% of dimethylsulfoxide (DMSO) to decrease the polarity of the medium, 5% of polyvinylpyrrolidone (PVP) to protect the enzyme against oxidation by quinones and 0.1mM of phenylmethylsulfonyl fluoride (PMSF) to protect the enzyme against degradation by protease. After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with the buffer system at a ratio of 1:1 (cells/buffer, *w/v*), together with each of the different protective agents. After filtration through two layers of muslin, 0.5ml of the resulting cell free extract was transferred into a 1.5ml Eppendorf centrifuge tube and the assay performed as described in 2.7.3, except that the reaction time was 60min.

It can be seen from Table 3.9. that 0.25M sucrose has no effect on enzyme activity, whilst 1% DMSO is inhibitory. However, the addition of 5% PVP or 0.1mM PMSF to the reaction mixtures completely inactivated the enzyme(s). These results show that the enzyme(s) require a mild hydrophobic environment, as an appropriate decrease in medium polarity and ionic strength by the addition of 0.25M sucrose did not affect the activity of the enzyme. However, excessive depolarisation caused by the addition of

DMSO partially inactivated the enzyme(s), because DMSO is normally used in more drastic cases (Cooper, 1977a). Oxidation caused by the presence of quinone compounds will be prevented by the addition of PVP, however, in this case, 5% PVP was found to inhibit the enzyme. PMSF, a specific protease inhibitor which has been used to inhibit some proteases (Cooper, 1977a), removed all enzyme activity at a concentration of 0.1mM. It would seem, according to these results, that none of the additives at the concentration used appeared to protect the enzyme(s) during the disruption of cultured cells, although 0.25M sucrose has no effect on enzyme activity. It may be useful in gradient centrifugation for subcellular fractionation and enzyme purification, or when frozen enzyme preparations are employed.

Table 3.9. Protective Effect of Selected Chemicals on Enzyme Activity During Cell Disruption

Addition*	Enzyme activity (^{14}C-nornicotine μmolx10^{-5}/tube)
Control	1.07 ± 0.21
None	6.98 ± 1.06
0.25M sucrose	6.26 ± 0.12
1% (v/v) DMSO	3.93 ± 0.25
5% (w/v) PVP	0.05 ± 0.28
0.1mM PMSF	0.00 ± 0.34

** Control was the same as 'none' except that it was boiled for 2 min. before the substrate was added. All values, after subtracting the control, are the means ± S.D. of three replicates.*

3.5.4. Solubilization of the enzyme(s)

3.5.4.1 Effects of selected detergents and NaCl on solubilization of the enzyme(s)

Solubilization is required if the enzyme protein is to be purified, because all the isolation procedures commonly used are in aqueous solution. In order to dissociate the enzyme which is presumably bound to, or associated with, the cell wall or cytoplasmic membranes, three types of detergent and 0.5M NaCl were examined for their ability to solubilize the enzyme(s). The three detergents tested were Triton X100 (non-ionic detergent), CHAPS and deoxycholate (anionic detergent). After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with 0.1M Tris-HCl buffer (pH9.0) supplemented with 3mM DTT and 5mM EDTA at a ratio of 1:1 (cells/buffer, *w/v*), together with each of the different detergents or NaCl. Following filtration through two layers of muslin, 0.5ml of the resulting cell free extract was then transferred into a 1.5ml Eppendorf centrifuge tube and the assay performed as described in 2.7.3, except that the reaction time was 60min. Three replicates were employed for all treatments and the control and the results are shown in Table 3.10.

Table 3.10. Solubilization of the Enzyme with Different Detergents and Salt

Substance used	Yield of protein* (mg/ml extracts)	Enzyme activity (^{14}C-NN μmolx10^{-5}/tube)
Control **	0.51±0.16	1.19±0.14
None	0.51±0.16	6.97±0.12
0.1% (*v/v*)Triton X100	1.76	1.45
0.3%(w/v)CHAPS	0.55±0.12	0.09±0.26
0.1%(w/v)Deoxycholate	0.58±0.20	0.00±0.18
0.5M NaCl	0.54±0.17	0.05±0.18

** Yield of protein presented without subtraction of the control.*
*** Control was as for 'none' except that it was boiled for 2min before the addition of the substrate; NN = nornicotine.*
The values for enzyme activity, after subtracting the control, are the means ± S.D. of three replicates.

The results in Table 3.10 show that virtually no ^{14}C-nornicotine was detecteded in the presence of these detergents and 0.5M NaCl. Although 0.3% of CHAPS, a zwitterionic detergent, has been reported to dissociate with high efficiency a *N*-methyltransferase involved in vindoline biosynthesis in the thylakoid membranes of chloroplasts (Dethier & De Luca, 1993). It was also observed that the addition of these detergents to the assay buffer did not increase the yield of protein except in the case of 0.1% Triton X100. However, Triton X100 made the chloroform fraction so sticky during extraction that the alkaloids could not be separated effectively on TLC; it also seriously affected the protein assay. These results show that the addition of detergents or NaCl at the amounts tested did not improve the enzyme assay.

3.5.4.2 Effect of pH of extraction buffer on solubility of protein

In order to investigate whether an alteration to the pH of the extraction buffer would improve the solubility of protein during disruption (see 2.7.2), 0.1M Tris-HCl buffer at different pHs, supplemented with 3mM DTT and 5mM EDTA, was used. After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with the buffer system at six different pHs at a ratio of 1:1 (cells/buffer, *w/v*). Following filtration through two layers of muslin, 20µl of the resulting cell-free extract was assayed for protein as described in 2.7.4.

The results in Fig.3.14 show that the pH of the extraction buffer affects the yield of soluble protein. A rise in alkalinity of the buffer increase the yield of protein until a maximum value of about 0.53mg/ml extract is reached at pH9.0. This result coincides with the maximum activity of the enzyme(s) at pH9.0 (see Table 3.8 and Fig.3.13). The effect of increasing pH of the extraction buffer on the yield of protein may be due to the fact that pH alters the binding of the protein to the cell wall and cell membranes, stimulating the release of associated proteins into the soluble phase.

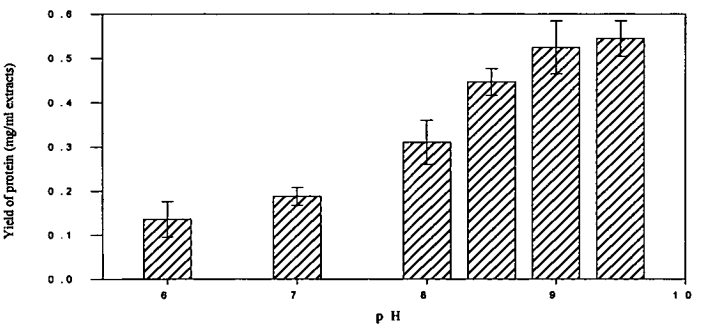


Fig. 3.14 Effect of pH on yield of soluble protein

Each point is the mean of three replicates ± *S.D.*.

3.5.4.3. Effect of centrifugation on release of the associated proteins

If the enzyme(s) is loosely associated with the cell wall or membranes, then it might be released further by extended centrifugation. To carry out this experiment, 0.1M Tris-HCl buffer (pH9.0) was used as before supplemented with 3mM DTT and 5mM EDTA. After removal of the 10 day old cells from the medium by filtration under reduced pressure, the cells were homogenised with the buffer system at a ratio of 1:1 (cells/buffer, *w/v*) and filtered through two layers of muslin. The resulting cell free extract was divided into four aliquots which were then subjected to centrifugation at 8,000*g* for 0min, 1min, 5min and 10min (one centrifugation time for each aliquot) using an Eppendorf centrifuge. Finally, the supernatants obtained were assayed as described in 2.7.3 except that the reaction time was 60min. Three replicates were employed for both treatments and controls.

It can be seen from the results presented in Fig.3.15 that the yield of soluble protein can be raised significantly by increasing the centrifugation time up to 10min. However, maximum enzyme activity of about 9.4×10^{-5}μmol was observed after centrifugation for only 1min. Clearly, increasing the centrifugation time is

accompanied by a decrease in enzyme activity. These results show that enzyme activity can be improved by centrifuging crude homogenates but only up to 1min. This may be due to the removal of some competitive proteins or inhibitors associated with the cell debris. Prolonged centrifugation might release these 'inhibitors' from the debris by increasing their solubility. In addition, machine temperature produced by prolonged centrifugation may cause some denaturation of the enzyme, since a conventional Eppendorf centrifuge was used in a cold room at 4°C.

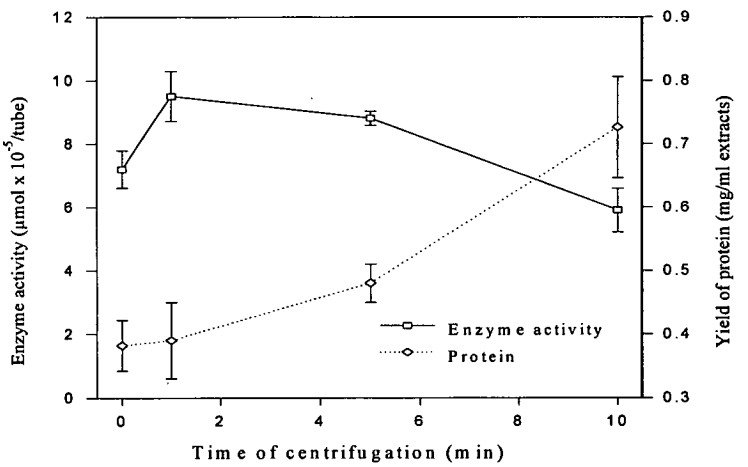


Fig.3.15 Effect of Centrifugation on Yield of Soluble Protein and Enzyme Activity

Each point is the mean of three replicates ± S.D..

In the next experiment, the distribution of the enzyme in crude cell-free preparations was investigated using the partially developed procedure.

3.5.5. Distribution of the enzyme(s) in crude cell-free extracts

According to the results already presented, the use of 0.1M Tris-HCl buffer (pH9.0) supplemented with 3mM DTT and 5mM EDTA with the preparation procedure set

out in 2.7.2 is satisfactory but probably not optimal. In order to determine which fraction the enzyme(s) occupies in the crude cell-free preparations, a study was made of the distribution of the enzyme(s). The 10 Day old cells were used and, as previously, all subsequent operations were performed in a cold room at 4°C. After removal of the cells from the medium by filtration under reduced pressure, the cells were homogenised with the buffer system at a ratio of 1:1 (cells/buffer, *w/v*) and filtered through two layers of muslin. A small sample of the resulting homogenate (about 2ml) was retained for assay (starting 'crude extract') and the rest slurry centrifuged at 8,000*g* for 1min to obtain the pellet and supernatant. After separation of the pellet from the supernatant by pipetting, the pellet was resuspended in about 3ml of preparation buffer. Finally, 0.5ml of the solution from the supernatant and the resuspended pellet was transferred into a 1.5ml Eppendorf centrifuge tube and assay performed as described in 2.7.3, except that the reaction time was 60min. Three replicates were used in all treatments and the control.

Table 3.11. Distribution of Enzyme Activity in Fractions of a Crude Cell-free Homogenate

Fraction	Yield of protein* (mg/ml.extracts)	Specific enzyme activity (ρKat×10^{-3}/mg.protein)
Control**	0.39±0.06	15.24±2.99
Crude extracts	0.39±0.06	99.34±15.10
Supernatant	0.41±0.11	138.89±5.83
Pellet	0.04±0.06	27.02±1.05

** Yield of protein is presented without subtraction of the control;*
*** Control is the crude extracts without centrifugation.*
The values of specific enzyme activity, after subtracting the control, are the means of three replicates ± S.D..

The results presented in Table 3.11 show that the maximal specific enzyme activity of 0.14ρKat per mg protein was found in the supernatant fraction. A specific activity of 27.02×10^{-3}ρKat per mg protein was detected in the pellet. These results could explain why many types of detergents (except Triton X100) and 0.5M of NaCl did not improve the solubility of the enzyme (see Table 3.10), as a majority of the enzyme is solubilised by the buffer used. The maximal enzyme activity in the supernatant is 9.1 times more than the control and superior to the crude extract. It can be seen that centrifugation of the crude homogenate for 1min increases the specific activity of the enzyme(s) by 40%.

3.5.6. Summary

The following points have emerged from the results obtained in this section and may now be summarized:

(1) Fresh 10 Day old cells were probably the best material for development of the enzyme assay;

(2) 0.1M Tris-HCl buffer (pH9.0), supplemented with 3mM DTT and 5mM EDTA, prepared with double-distilled water provided a satisfactory starting point for development of the assay;

(3) The addition of sucrose (0.25M), DMSO (1%), PVP (5%) and PMSF (0.1mM) to the extraction buffer during the cell disruption did not improve enzyme yield;

(4) The maximum amount of soluble protein was found in the extract made with 0.1M Tris-HCl buffer at pH9.0 after centrifugation at 8,800*g* for 1min;

(5) Most of the enzyme activity was present the supernatant fraction.

It has now been demonstrated for the first time that cell-free extracts from cell cultures of tobacco are able to convert added nicotine to nornicotine. According to the results obtained, an *in vitro* enzyme assay procedure has been developed as described in 2.7.

However, the mechanism of this reaction is still unclear. In view of the suggestion that the term, demethylation, can be used to describe two general reactions: transmethylation and demethylation (Poulton, 1981), this enzyme may now be tentatively designated as nicotine *N*-demethylase.

Using this developed assay system, attempts were now made to further optimise this assay system and to characterise the enzyme(s) in the cell-free preparations.

3.6. Characterisation of Nicotine *N*-Demethylase in Cell-free Extracts

Activity of nicotine *N*-demethylase, an enzyme catalysing the *N*-demethylation of nicotine to nornicotine, in crude cell-free extracts of cultured cells of *Nicotiana tabacum* L cv. Wisconsin-38 has now been established. An isotopic enzyme assay procedure has been developed. In this section, further attempts were made to characterise nicotine *N*-demethylase using cell-free preparations. It was expected that the information obtained would help in the purification of this enzyme and to determine the kinetics of this reaction. First of all, pH and temperature optima were determined.

3.6.1. Effect of pH on the activity of nicotine *N*-demethylase

The cell-free preparations were made (see Fig.2.5) and the enzyme assayed as described in 2.7, except that the pH was varied. The results in Fig.3.16 show maximal enzyme activity between pH9.0 and 9.5. This result coincides with the results presented in Fig.3.13.

3.6.2. Effect of temperature on the activity of nicotine *N*- demethylase

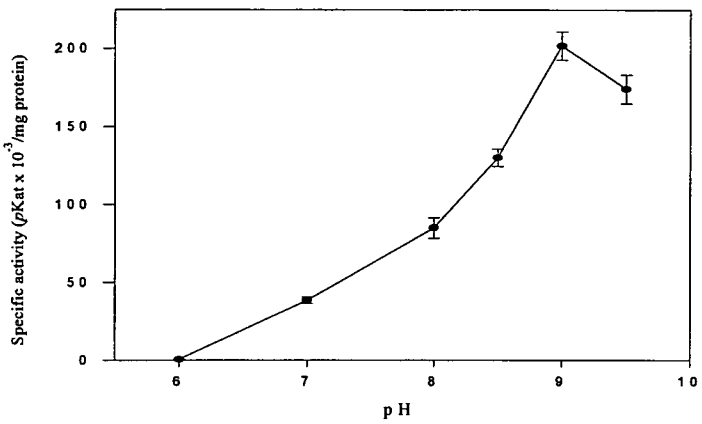


Fig.3.16 Effect of pH on the Activity of Nicotine *N*-Demethylase

Assay was performed using the optimum procedure except for pH. Reaction time was 30min and each value was corrected for the scintillation blank and assay control and the values presented are the means of three replicates ± S.D..

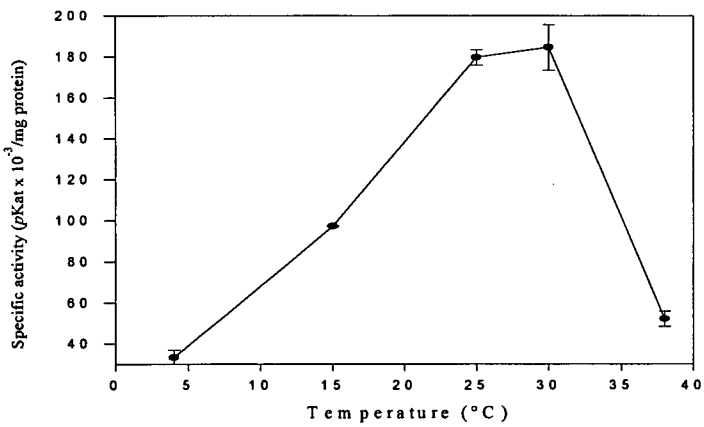


Fig. 3.17 Effect of Temperature on the Activity of Nicotine *N*-Demethylation

Assay was performed using the optimum procedure except for temperature. Reaction time was 30min and each value was corrected for the scintillation blank and assay control and the values presented are the means of three replicates ± S.D..

The cell-free preparations and the enzyme was assayed as described in 2.7, except that the reaction was carried out at a range of temperatures. From the result shown in Fig.3.17, it can be seen that the optimal temperature lies between 25°C and 30°C.

3.6.3. Effect of enzyme concentration on the production of ^{14}C-nornicotine from ^{14}C-nicotine

The aim of this experiment was to investigate the relationship between reaction velocity and enzyme concentration. The cell-free preparations were made (see Fig.2.5) and enzyme assayed as described in 2.7., except that the amount of enzyme was varied. A fixed amount of ^{14}C-nicotine substrate (3.8×10^{-3}μmol) was used. Three replicates were used throughout for treatments and the controls. It can be seen from the results presented in Fig.3.18 that a linear relationship appears to exist between 0.11mg and 0.22mg of enzyme concentration.

The next step in this study was to establish a time course for this reaction at pH9.0 and 30°C with an enzyme concentration between 0.22-0.44mg/ml.

3.6.4. Time course of ^{14}C-nornicotine production from ^{14}C-nicotine catalysed by nicotine *N*-demethylase

Enzyme was assayed as described in 2.7, except that the reaction time was varied. It can be seen from Fig.3.19 that the enzyme activity increases sharply for up to 30min when it remains constant until the end of the 90min time course. Accordingly, the kinetic studies on the reaction in the next experiment were carried out with a fixed incubation time of 30min.

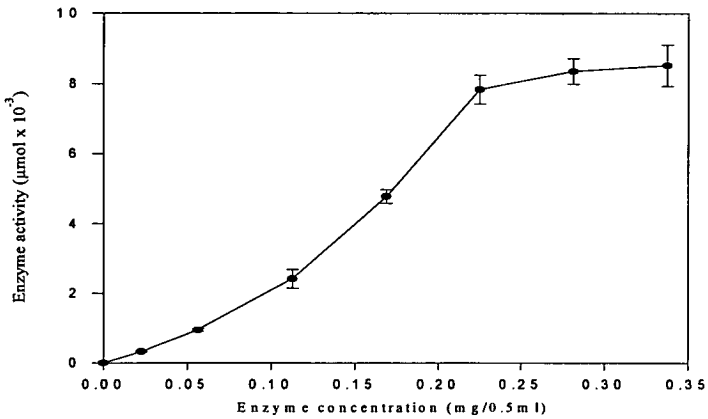


Fig.3.18 Effect of Concentration of Nicotine *N*-Demethylase on Enzyme Activity

Assay was performed using the procedure already reported except for enzyme concentration. Reaction time was 30min and each value was corrected for the scintillation blank and assay control and the values presented are the means of three replicates ± S.D..

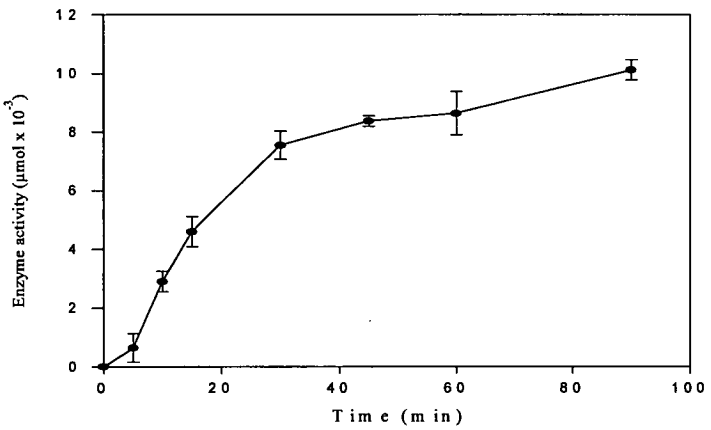


Fig. 3.19 Time Course of ^{14}C-Nornicotine Production Catalysed by Nicotine *N*-Demethylase

Assay was performed using the optimum procedure except for reaction time. Each value was corrected for the scintillation blank and assay control and the values presented are the means of three replicates ± S.D..

3.6.5. Effect of substrate concentration on the velocity of the reaction

The cell-free preparations were made (see Fig.2.5) and the enzyme assayed as described in 2.7., except that the ^{14}C-nicotine substrate concentration was varied. Three replicates of treatments and the controls were used throughout. The relationship between substrate concentration and reaction velocity is shown in Fig.3.20. It can be seen that velocity increases sharply, accompanying the increase in substrate concentration for up to 7.6μM (corresponding to 3.8×10^{-3}μmol of ^{14}C-nicotine) whereafter the velocity remains constant regardless the increase of ^{14}C-nicotine concentration. Taking reciprocals of the two sets of data, the Lineweaver-Burk equation (Wilson, 1986) was regressed with a 99.2% coefficient of determination, using a statistical computing package (Minitab, USA), as shown in Fig.3.21. From this, the Michaelis constant (Km) of 7.39μM and a maximum reaction velocity (Vmax) of 7.58×10^{-2}ρKat were obtained (see Fig.3.20).

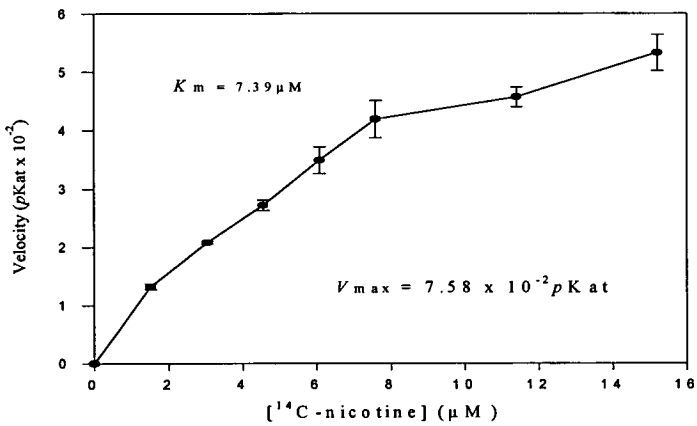


Fig.3.20 Effect of Concentration of ^{14}C-Nicotine on Reaction Velocity

Assay was performed using the optimum procedure except for the amount of ^{14}C-nicotine. Reaction time was 30min and each value was corrected for the scintillation blank and assay control and the values presented are the means of three replicates ± S.D..

According to the characteristics of the enzymatic reaction obtained above, further attempts were made to determine the effects of co-factors, co-enzymes and some possible methyl group acceptors on the activity of nicotine *N*-demethylase. The assay was performed at pH 9.0 and 30°C with a fixed reaction time of 30min, a substrate concentration of 3.8×10^{-3}μmol and a enzyme concentration between 0.11mg and 0.22mg per reaction tube.

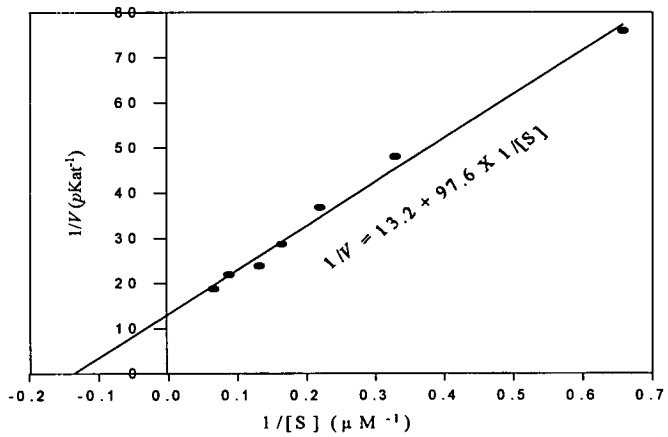


Fig.3.21 Linear Plot of the Lineweaver-Burk Equation for the Calculation of *K*m and *V*max

3.6.6. Effect of co-factors and co-enzymes on the activity of nicotine *N*-demethylase

In order to determine whether this *N*-demethylation is dependent on co-factors or co-enzymes, several common co-factors and co-enzymes were added to the reaction mixture. The constituents and conditions of the assay were as described in 2.7. and three replicates were used for all treatments and the controls. The results are presented in Table 3.12.

It can be seen from Table 3.12 that the addition of 0.5mM ATP, 1mM β-NAD or 1mM β-NADP to the assay does not affect enzyme activity, whilst 1mM β-NADH

slightly decreased enzyme activity. However, 1mM β-NADPH increased enzyme activity slightly. These results suggest that this enzyme(s) does not need added ATP, β-NAD or β-NADP but may require the reduced form of β-NADP (β-NADPH). Since a crude cell-free enzyme preparation was used in this experiment, it is difficult to say whether this enzyme(s) is β-NADPH dependent. Further study using a purified enzyme preparation would be necessary to establish dependency on β-NADPH.

Table 3.12. Effects of Co-factors and Co-enzymes on Enzyme Activity

Addition*	Amount added (mM)	Specific enzyme activity (ρKat×10^{-3}/mg.protein)
Control	---	15.42 ± 1.89
None	---	206.76 ± 20.77
ATP	0.5	245.76 ± 27.05
β-NADPH	1.0	285.99 ± 37.92
β-NADH	1.0	170.77 ± 7.49
β-NADP	1.0	206.28 ± 5.31
β-NAD	1.0	206.52 ± 24.40

** All co-factors and co-enzymes were added to the reaction mixture at the same time as the substrate; Control was the same as the treatments except that it was boiled for 2min before the addition of the substrate;*
*** Specific activity was defined as the proportion of total enzyme catalytic activity (pKat) to the total amount of protein (mg) present in the reaction mixture. Each value, after subtracting the control, is the mean of three replicates ± S.D..*

3.6.7. Is this reaction a transmethylation?

This is the question that has long been unanswered about nicotine bioconversion, in which a methyl group on the pyrrolidine ring moiety of the nicotine molecule disappears. There is no substantial evidence, however, for or against the possibility

of a transmethylation which was presumed by Wallex & Nowacki (1978) and James (1975). In order to establish whether this conversion is a *N*-transmethylation, the effects of selected methyl group acceptors on the conversion of nicotine to nornicotine in a cell-free system were studied.

Various amounts of putrescine, glycine and ethanolamine were added to the reaction mixture together with the ^{14}C-nicotine substrate. The overall enzyme assay process was the same as described in 2.7 and three replicates were used for all treatments and the controls. The results are presented in Table 3.13.

Table 3.13. Effects of Some Possible Methyl Group Acceptors on Enzyme Activity

Methyl group acceptor*	Amount added (mM)	Specific enzyme activity (ρKat×10^{-3}/mg.protein)
Control	---	12.05 ± 1.16
None	---	244.18 ± 6.94
Putrescine	3.0	128.30 ± 5.24
	0.3	156.80 ± 4.20
Glycine	0.1	200.80 ± 8.34
	0.01	239.80 ± 25.56
Ethanolamine	100	25.40 ± 3.73
	5	148.50 ± 2.90

** All selected methyl group acceptors were added to the reaction mixture at the same time as the substrate;*
*** Specific activity was defined as the proportion of total enzyme catalytic activity (ρKat) to the total amount of protein (mg) present in the reaction mixture;*
Control was made as for the treatments except that it was boiled for 2min. Each values, after subtracting the control, is the mean of three replicates ± S.D..

None of the three possible methyl group acceptors, putrescine, glycine and ethanolamine, showed a positive effect on enzyme activity, when these compounds was added to the assay at a range of concentrations. Whilst 100mM ethanolamine denatured nicotine *N*-demethylase. The results obtained do not support the suggestion that this reaction is a *N*-transmethylation. However, this can not be ruled out at this stage.

3.6.8. Summary

Using the enzyme assay procedure developed in 3.5, the characteristics of nicotine *N*-demethylase in a crude cell-free enzyme preparation can be summarised as follows:

(1) The pH optimum is between 9.0 and 9.5;
(2) The temperature optimum is between 25°C and 30°C;
(3) The reaction is proceeding effectively for up to 30min when it remains constant;
(4) The relationship between substrate concentration and reaction velocity is more or less linear up to 7.6μM of ^{14}C-nicotine; the Michaelis constant (K_m) and the maximum rate (V_{mas}) of this reaction are 7.39μM and 7.58×10^{-2}ρKat respectively;
(5) There is a linear relationship between enzyme concentration and ^{14}C-nornicotine production between 0.11mg and 0.22mg;
(6) The addition of 0.5mM ATP, 1mM β-NAD or β-NADP to the assay did not affect enzyme activity, whilst 1mM β-NADPH increased the activity slightly;
(7) None of three selected methyl group acceptors, putrescine, glycine and ethanolamine, showed a positive effect on enzyme activity.

Having partially characterised nicotine *N*-demethylase in a crude cell preparation, attempts were now made in the next section to study the pattern of enzyme activity

during a culture cycle and the effect of light on enzyme activity. Using isopycnic differential centrifugation, the subcellular distribution of this enzyme(s) was determined in detail together with the nature of each subcellular fraction using TE microscopy. In addition, dialysis and gel filtration of the enzyme preparation were carried out to further characterise this enzyme(s) and preliminary enzyme purification was also attempted.

3.7. Studies on the Enzymology of Nicotine Demethylation to Nornicotine

The aim of this set of experiments was to study the enzymology of nicotine to nornicotine. It has been reported that this *N*-demethylation might be associated with photosynthesis either directly (Manceau, et al. 1989) or indirectly (Hobbs & Yeoman, 1991a). In order to examine this possibility, an attempt was made to compare the activity of nicotine *N*-demethylase throughout a 20 day culture cycle in cells maintained in the dark and those in the light. Also, an attempt was made to explore the subcellular localisation of nicotine *N*-demethylase using isopycnic differential centrifugation. Subsequently, the fraction containing the highest enzyme activity was purified by dialysis and gel filtration. The semi-purified enzyme preparation was then used to further determine the characteristics of this enzyme(s). In particular, the possible role of selected methyl group acceptors was studied. The results obtained also provided further information to enable the purification of nicotine *N*-demethylase. First of all, the time course of enzyme activity in dark-cultured cells was studied.

3.7.1. Time course of activity of nicotine *N*-demethylase in dark-cultured cells

In this experiment, a time course of the activity of nicotine *N*-demethylase in dark-cultured cells over a 20 day culture cycle was determined using the enzyme assay procedure already reported. The cell cultures and cultural conditions used were the same as those described in 2.1 and 2.2. The cells were harvested every 2 days from day 0 to day 20, with three replicates (flasks) for each sample. Following the removal of cells from medium by filtration under pressure, 2.0g fresh weight of cells was used from each of the sampling times. However, all the cells from the 0, 2 and 4 day cultures were used, because the total fresh weight for these times was less than 2g. The enzyme preparations were made and the activity assayed as described in 2.7.2 and 2.7.3.

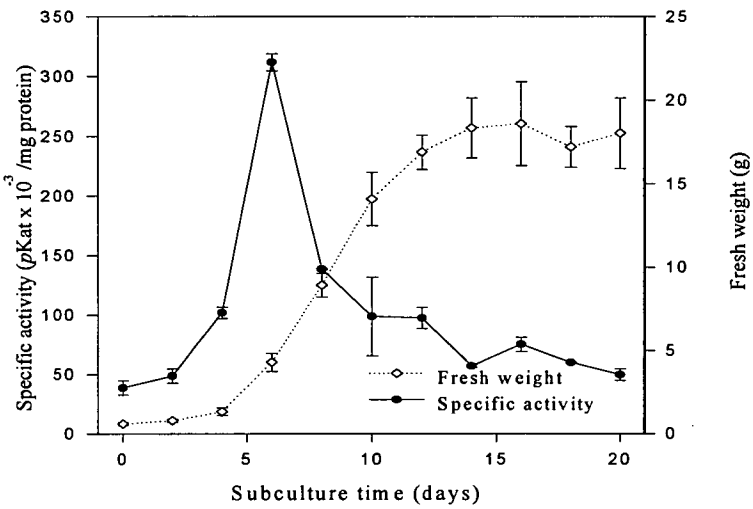


Fig.3.22 Time Course of the Specific Activity of Nicotine *N*-Demethylase in Dark-cultured Cells

Enzyme assay was performed using the method described in 2.7. Each value was corrected for the scintillation blank and assay control and the values presented are the mean of three replicates ± S.D..

The time course of nicotine *N*-demethylase activity in dark-cultured cells over a 20 day culture cycle is shown in Fig.3.22 from which, it can be seen that there is considerable variation in enzyme activity over the culture cycle. In the first two days, there is only a small amount of activity in the cells. Subsequently, when the growth of the cultures enters a period of linear increase at day 4, the activity of nicotine *N*-demethylase increases sharply, reaching a maximum specific activity of about 0.31ρ Kat per mg.protein at around day 6. From day 6 to day 10, the specific activity drops rapidly, while the growth of the cultures is still increasing. Thereafter, both the growth and the activity gradually become constant and remain so until the end of the 20 day culture cycle.

The results obtained are consistent with those from previous experiments (see Fig.3.3), in which the maximal amount of nornicotine produced per gram fresh weight occurred at day 5 in the 20 day culture cycle. In the next experiment, the effect of light on the activity of nicotine *N*-demethylase during a culture cycle was studied.

3.7.2. Effect of light on the activity of nicotine *N*-demethylase during a culture cycle

Having established that the maximum activity of nicotine *N*-demethylase occurs around day 6 of a 20 day culture cycle in the dark-cultured cells (see Fig.3.22), it was decided to discover whether light would affect the pattern of nicotine *N*-demethylase activity. Accordingly, similar cultures to those used in 3.7.1 were incubated in continuous light (Compton Warmwhite Fluorescent, 15±3μmol sec^{-1} m^{-2}). The other conditions were the same as described in 2.2. After sub-culture every 20 days for three culture cycles, the cells turned green (cells cultured in the dark were normally

pale yellow). These light-cultured cells were then used to determine the activity of nicotine *N*-demethylase in a 20 day culture cycle as described in 3.7.1.

The results presented in Fig.3.23 show that there is a slow increase in the specific activity of nicotine *N*-demethylase during the first 4 days of the 20 day culture cycle, followed by a sharp increase in enzyme activity beginning around day 5. The increase parallels the linear increase in fresh weight of the cultures, with maximum values of specific enzyme activity of ca. 0.39ρKat per mg.protein by day 10. Thereafter, enzyme activity dropped gradually, while the fresh weight of the cultures remained constant until the end of the culture cycle.

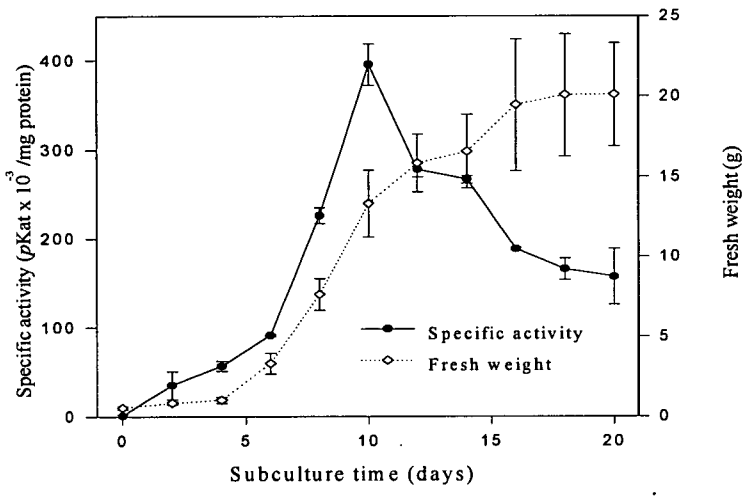


Fig.3.23 Effect of light on the Specific Activity of Nicotine *N*-Demethylase in a 20 day culture cycle

Enzyme assay was performed using the method described in 2.7. Each value was corrected for the scintillation blank and assay control and the values presented are the mean of three replicates ± S.D..

In comparison with the results shown in Fig.3.22, it would appear that the maximal activity of nicotine *N*-demethylase in the light-cultured cells in a 20 day culture cycle occurs in the late period of linear growth, not in the early period of linear growth as

in dark-cultured cells, whilst the growth pattern of both these cultures is very similar. In other words, the point at which maximal enzyme activity is reached in dark-cultured cells is about 4 days earlier than that in light-cultured cells. In addition, there is about a 26% increase in the maximal specific activity of nicotine *N*-demethylase in light-cultured cells, compared to dark-cultured cells. The results suggest that light may delay the maximal point of enzyme activity in a culture cycle, but increase the overall activity of nicotine *N*-demethylase. This suggests that there might be some relationship between the photosynthetic system and bioconversion activity in some cell lines, since a mixotrophic cell line was used in these investigations.

In the next experiment, attempts were made to reveal the subcellular distribution of nicotine *N*-demethylase in dark-cultured cells by using differential centrifugation and TEM.

3.7.3. Sub-cellular localisation of nicotine *N*-demethylase in crude homogenates of cultured cells

The aim of this experiment was to determine the sub-cellular localisation of nicotine *N*-demethylase in the cell. Such information should prove useful in further characterisation of the enzyme(s) and, also, in the establishment of a protocol for enzyme purification.

According to the result presented in 3.7.1 maximal activity of nicotine *N*-demethylase occurs at around day 6 of a 20 day culture cycle. 10g of fresh cells from a 7 day old culture (see 2.8 for justification for using 7 day old cells) was selected. A small amount of the cells was first fixed in 2% glutaraldehyde in 50mM sodium cacodylate (pH7.2) for TEM and the remainder homogenised in 10ml of 0.1M Tris-

HCl buffer (pH9.0), supplemented with 5mM EDTA, 3mM DTT and 0.25M sucrose, as described in 2.8. The crude homogenate was then divided into two parts and, to one of the aliquots an equal volume of 2% glutaraldehyde in 50mM sodium cacodylate (pH7.2) was added. 2ml of the other aliquot was retained for assay (original homogenate). Both of these aliquots were then centrifuged at 1,000*g* for 10min to yield supernatant 1 (S1) and the cell wall pellet. Further centrifugation of S1 at 13,000*g* for 15min produced supernatant 2 (S2) and the intermediate pellet. Subsequently, S2 was centrifuged at 100,000*g* for 1hr to obtain the microsomal pellet and the soluble fraction. The pellets with fixative and the fixed intact cells were processed as described in 2.9, whilst those pellets without fixative from each of the centrifugations were re-suspended in 2ml of the preparation buffer. Finally, 0.5ml of each subcellular fraction together with the original homogenate was subjected to enzyme and protein assay as described in 2.7.3 and 2.7.4. The results for total activity, protein and specific activity of each subcellular fraction are given in Table 3.14.

7 day old cultured cells were homogenised and fractionated by differential centrifugation into four fractions and the results presented in Fig.3.24. It can be seen that intact cells (A) from this culture exhibit a typical morphological appearance, with abnormal cell shapes, a large central vacuole occupying the majority of the cell volume and thin peripheral cytoplasm (including a nucleus) lining the wall. After homogenisation and subsequent centrifugation (1,000*g* for 10min), a fraction rich in cell-wall fragments was obtained (B). Further centrifugation (13,000*g* for 15min) produced an intermediate fraction, in which there are many spherules, large membrane vesicles and other unidentified structures. Final centrifugation at 100,000*g* for 1hr yielded a microsomal pellet (C) containing a few small membrane vesicles and membrane fragments, and the supernatant fraction (D).

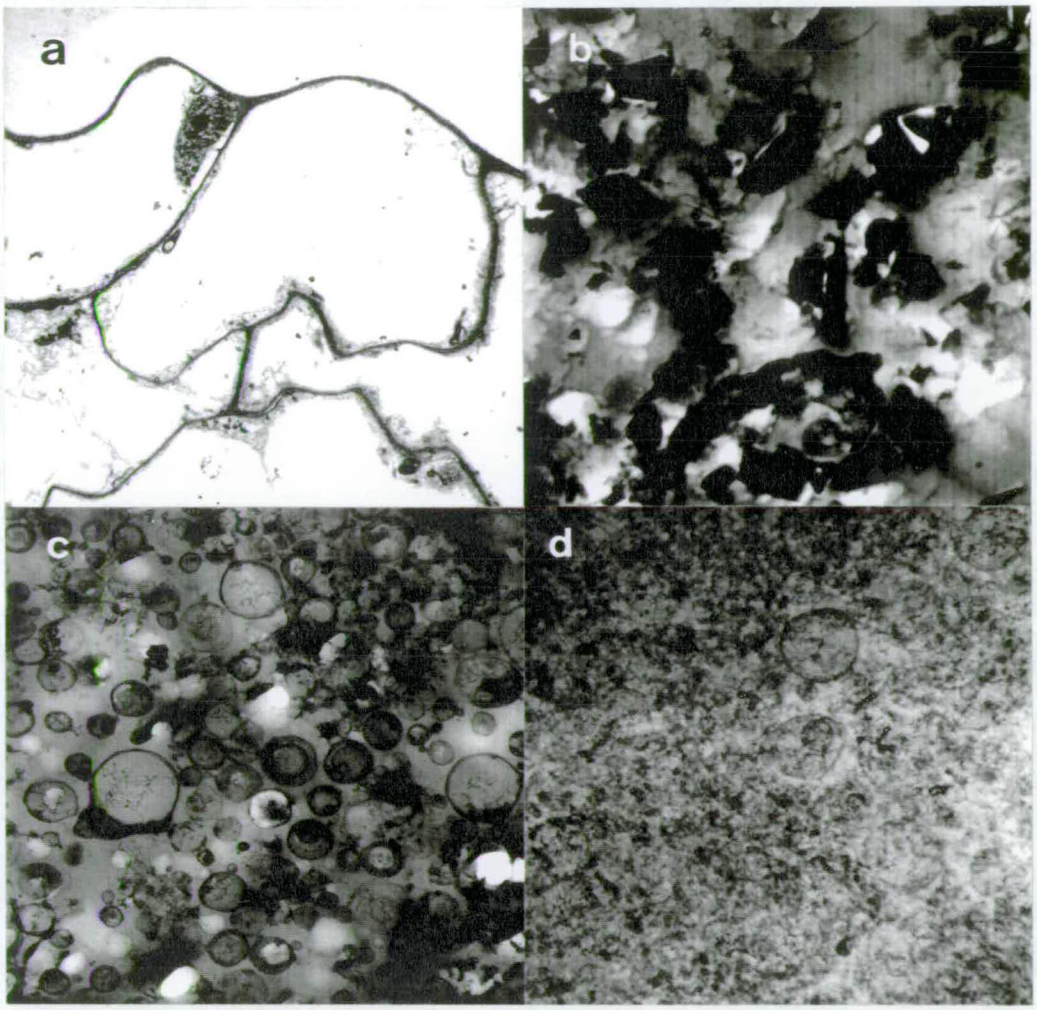


Fig.3.24 Subcellular Fractionation of 7 Day Old Tobacco Cell Culture

(a): Section of small aggregates of intact cells; magnification ×1,000. (b): Cell wall pellet; magnification ×5,000. (c): Intermediate fraction; magnification ×15,000. (d): Microsomal pellet; magnification ×20,000. Details of the fractionation and TEM method are given in 2.8 and 2.9 respectively.

The results presented in Table 3.14 show that nicotine *N*-demethylase activity was recovered in all fractions. The enzyme activity varied from fraction to fraction, with 65.3% of the total activity of the original homogenate present in the supernatant (S1)

fraction, and 22.8% of the activity associated with the cell wall pellet. The highest enzyme activity of 0.5ρKat (38.2%) of the four fractions was found in the intermediate pellet and, the microsomal and soluble fraction contain 3.6% and 8.0% of the total activity respectively. Of the four subcellular fractions, i.e. cell wall, intermediate, microsomal and soluble, a maximum specific activity of about 1.5ρKat per mg.protein was associated with the microsomal fraction, although a relatively high specific activity of 0.9ρKat per mg.protein was found in the intermediate fraction. Only 0.14 and 0.1ρKat per mg.protein were associated with the cell wall and soluble fraction respectively.

Table 3.14. Subcellular Fractionation of Nicotine *N*-Demethylase

Fraction	Total activity (10^{-2}ρKat)	Protein (mg)	Specific enzyme activity (ρKat×10^{-2}/mg.protein)
Crude homogenate	123.2 ± 3.3 (100%)	5.04 (100%)	20.4 ± 0.6
S1.	80.4 ± 4.5 (65.3%)	3.42 (67.9%)	23.5 ± 1.3
Cell wall	28.1 ± 1.0 (22.8%)	2.08 (41.3%)	13.5 ± 0.5
S2.	13.4 ± 2.5 (10.9%)	1.68 (33.3%)	8.0 ± 1.5
Intermediate	47.1 ± 0.4 (38.2%)	0.52 (10.3%)	90.6 ± 0.8
Soluble (S3.)	9.9 ± 0.4 (8.0%)	1.0 (19.8%)	9.9 ± 0.4
Microsomal	4.4 ± 0.6 (3.6%)	0.03 (0.6%)	146.7 ± 20.0

After correction for the scintillation blank and assay control, each value is either the mean (for protein) or mean of three replicates±S.D.. Values given in parentheses are either enzyme activity or protein recovered as a percentage of the starting crude homogenate.

It would appear from the results presented that nicotine *N*-demethylase is not tightly associated with the cell wall, as the predominant activity was present in the supernatant. Nicotine *N*-demethylase is divided between the intermediate and the microsomal fraction, in which some membrane vesicles and/or fragments may be associated with this activity. However, further investigation, using density gradient centrifugation or an immunochemical technique, is required to ascertain the precise subcellular localisation, since differential centrifugation is subject to cross-contamination.

In the next set of experiments, attempts were made to further characterise nicotine *N*-demethylase using dialysed enzyme preparations.

3.7.4. Studies on nicotine *N*-demethylase using a dialysed enzyme preparation

In previous experiments (see 3.6), it has been shown that the activity of nicotine *N*-demethylase was not enhanced by the addition of 0.5mM ATP, 1mM β-NAD or β-NADP, whilst 1mM β-NADPH did increase enzyme activity. In addition, none of the three methyl group acceptors selected, putrescine, glycine or ethanolamine, displayed a positive effect on enzyme activity. However, it is not known whether the endogenous amount of these co-enzyme, co-factors or the possible methyl group acceptors which may be in the crude homogenate are present at an optimal level. It is well known that dialysis will remove not only ions but also small metabolites such as ATP and co-enzymes (Cooper, 1977a). Accordingly, the employment of a dialysed enzyme preparation will enable the examination of a range of co-factors and co-enzymes on the activity of nicotine *N*-demethylation.

It has already been demonstrated that most of the nicotine *N*-demethylase activity is present in the supernatant (S1) (see 3.7.3). Accordingly, 15ml of the supernatant (S1) obtained by centrifugation of the crude homogenate at 1,000*g* for 10min was selected and dialysed overnight against the preparation buffer. The overall procedure was as described in 2.10. In order to distinguish between denaturation effects on the enzyme caused by storage in the cold for 24hr from that of dialysis, an experimental control (S1') without dialysis, the same as S1 was maintained beside the dialysis beaker under the same conditions and assayed 24hr later together with the dialysed samples. After dialysis, 0.5ml of the dialysed enzyme preparation was transferred into each 1.5ml Eppendorf tube, to which a varied amount of different co-enzymes or possible methyl group acceptors was added. Enzyme and protein assays were then performed as described in 2.7.3 and 2.7.4. The results are presented in Tables 3.15 and 3.16.

It can be seen from Table 3.15 that storage of the supernatant (S1) for 24hr resulted in a marked reduction in nicotine *N*-demethylase activity to 0.1ρKat/mg.protein activity in S1', compared with 0.2ρKat/mg.protein in fresh S1. It was also shown that the activity of dialysed S1 (Dia-S1) was even less than S1'. This was probably due to the combinative fact of denaturation of enzyme during the 24hr dialysis and the removal of some small endogenous molecules necessary for enzyme activity.

In comparison with Dia-S1, the addition of co-factors or co-enzymes to the dialysed enzyme solution increased enzyme activity in all cases but to different extents. The addition of 0.5mM ATP and 1.0mM NAD and NADP to Dia-S1 restored enzyme activity to the level of S1', showing that dialysis of S1 had removed some of the endogenous molecules present in the enzyme preparation. Although the addition of these molecules (ATP, NAD and NADP) to dialysed S1 (Dia-S1) restored enzyme activity to S1' level, no spectacular increase in enzyme activity was detected. It

would appear that the enzyme(S) is unlikely to be ATP, NAD and NADP dependent, but these co-factors or co-enzymes may affect the enzyme activity through other metabolic pathway(s) which are associated with this enzymatic reaction. In contrast, it can be seen from Table 3.15 that the addition of the reduced form NAD and NADP to Dia-S1 resulted in a large increase in enzyme activity. In particular, there was a considerable improvement in enzyme activity (0.8ρKat/mg.protein) when 1.0mM NADPH was added to Dia-S1, although 1.0mM NADH also increased enzyme activity. The results obtained agree with those already presented (see 3.6.6, Table 3.12), suggesting that this enzyme(s) is NADPH dependent.

Table 3.15. Effects of Co-factors and Co-enzymes on the Activity of Nicotine *N*-Demethylase in a Dialysed Enzyme Solution

Addition	Amount added (mM)	Specific enzyme activity (ρKat×10^{-3}/mg.protein)
Control	---	10.4 ± 1.9
Fresh S1.	---	212.0 ± 23.4
S1'.	---	94.9 ± 1.6
Dia-S1.	---	54.2 ± 2.4
ATP	0.5	93.4 ± 15.3
β-NADPH	1.0	602.8 ± 28.5
β-NADH	1.0	188.5 ± 18.6
β-NADP	1.0	99.0 ± 9.1
β-NAD	1.0	81.0 ± 9.0

S1 (the supernatant (S1) as shown in Fig.2.5), S1' (as S1 but not dialysed) and the dialysed S1 (Dia-S1) were all assayed without the addition of any of the co-enzymes and co-factors. All additives were added to the reaction mixture at the same time as the substrate. Control was the same as the samples except that it was boiled for 2min before the addition of substrate.

Each value is the mean of three replicates±S.D., after correction for the scintillation blank and assay control.

Using the dialysed supernatant (Dia-S1), three possible methyl group acceptors were tested for their effect on enzyme activity. The results presented in Table 3.16 show that none of these selected methyl group acceptors had a positive effect on enzyme activity. This result coincides with that obtained previously (see 3.6.7 and Table 3.13) indicating that this reaction appears unlikely to be a transmethylation.

Table 3.16. Effects of Some Possible Methyl Group Acceptors on the Activity of Nicotine *N*-Demethylase in a Dialysed Enzyme Solution

Methyl group acceptor	Amount added (mM)	Specific enzyme activity (ρKat×10^{-3}/mg.protein)
Control	---	10.4 ± 1.9
S1.	---	212.0 ± 23.4
Dia-S1.	---	54.2 ± 2.4
Putrescine	0.3	58.9 ± 11.0
Glycine	0.01	48.2 ± 0.6
Ethanolamine	5.0	27.9 ± 20.4

S1, the supernatant 1 as shown in Fig.2.5, and dialysed S1 (Dia-S1) were assayed without the addition of any of the selected methyl group acceptors. All additions were made to the reaction mixture at the same time as the addition of substrate;
Each value is the mean of three replicates ± S.D., after the correction for the scintillation blank and assay control.

Having refined the characteristics of nicotine *N*-demethylase, attempts were now made in the next set of experiments to study this enzyme(s) further using gel filtration.

3.7.5. Studies on nicotine *N*-demethylase using gel filtration

The aim of this set of experiments was to explore whether any molecules other than nicotine *N*-demethylase are involved in this bioconversion. This could provide evidence for the presence of participating small molecules such as methyl group acceptors. This was achieved by fractionation of crude enzyme preparations using Sephadex G-25 gel filtration. After establishment of a gel chromatographic pattern of enzyme activity in a crude enzyme preparation, each of the fractions containing no enzyme activity was added either to the fraction or combination of the fractions which contained enzyme activity. Any improvement in enzyme activity would suggest the involvement of small molecule(s) in nicotine *N*-demethylation. Accordingly, it was first necessary to establish a gel fractionation profile for enzyme activity from a crude enzyme preparation.

3.7.5.1 Fractionation pattern of enzyme activity on a Sephadex G-25 gel column

Having previously established that most of the activity of nicotine *N*-demethylase was present in the supernatant (S1) (see 3.7.3), this fraction was selected for the next part of the investigation. Preparation of the Sephadex G-25 column was as described in 2.1.1 and the overall process was carried out in a cold room at 4°C. A fresh 7 day old cell culture was used and the cells were separated from the medium by filtration under pressure. 6g of fresh cells was then homogenised with 6ml of chilled 0.1M Tris-HCl buffer (pH9.0), supplemented with 5mM EDTA and 3mM DTT, and the homogenate was centrifuged at 1,000*g* for 10min. The equilibration buffer (the preparation buffer) was run off from the chilled column just exposing the top surface of the gel bed, then 1ml of the supernatant from the 1,000*g* centrifugation was carefully loaded. A further 1ml of the buffer was released from the column to allow the sample to penetrate into the gel bed. Subsequently, the elution buffer was carefully added to the top of the column and to the extension tube to achieve a

gravitational flow rate of approximately 2ml/min. Finally, 18 samples of 0.75ml per tube were collected. The absorbance of each eluent was measured at 280nm, using a UV/Vis Spectrophotometer (Philips, PU8625 Series), to obtain an elution profile. Meanwhile, 0.5ml of each eluent was assayed for protein and enzyme activity as described previously.

The results presented in Fig.3.25A illustrate that material absorbing at 280nm appears in tube-1 and increases sharply until a maximum point was reached in tube-4. From tube-4 to tube-6, the absorbance decreased rapidly and, thereafter, remained relatively constant until the end of the elution. It would appear from the peak shape of absorbance at 280nm that the sample volume, viscosity, ion strength etc were compatible with gel particle size, bed volume and elution rate, ensuring a reliable elution profile for the separation of proteins from small molecules (see Cooper,1977b).

Protein and nicotine *N*-demethylase activity are illustrated in Fig.3.25B, and show that both protein and enzyme activity appear over a broad range from tube-2 to tube-6 exclusively, whilst maximal enzyme activity appeared at tube-3. Neither protein nor enzyme activity were detected in tube-1 and those after tube-6. In order to determine whether there were any small molecules other than the enzyme itself which might be involved in this *N*-demethylation, the contents of tube-3 and-4 were combined, to which each of the contents in the remaining tubes were added. The enzyme and protein in these mixtures were assayed to see whether enzyme activity would be improved over the combination of tubes-3 and 4. This was performed in the next experiment.

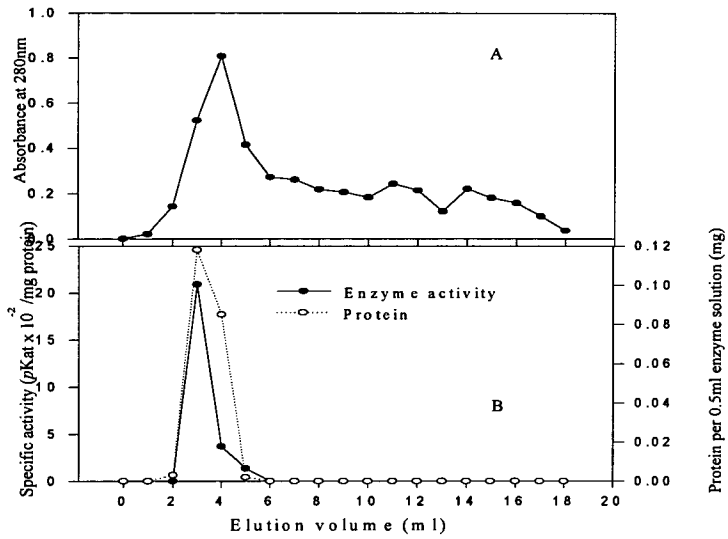


Fig.3.25 Fractionation Pattern of an Enzyme Preparation Chromatographed on a Sephadex G25 Column

A: 280nm absorbance; B: enzyme activity and yield of protein. Each value for enzyme activity was corrected for the scintillation blank and assay control and the values presented including those for protein and 280nm absorbance are the mean of three replicates.

3.7.5.2 Nicotine *N*-demethylase activity in the combined elution tube contents from Sephadex G-25 gel filtration

Having established in 3.7.5.1 that the maximal enzyme activity appeared in tube-3 of the Sephadex G-25 gel filtration profile (see Fig.3.25B), the contents of tubes-3 and 4 were combined and diluted with the original buffer to give a final volume of 4.5ml. Subsequently, 0.25ml of this combination was mixed (in 1.5ml Eppendorf centrifugation tubes) with 0.25ml of the contents from each of the uncombined elution tubes. Finally, these mixtures in a final volume of 0.5ml were assayed for both protein and enzyme as described previously.

Table 3.17. Nicotine *N*-Demethylase Activity in the Combined Elution Tubes Resulted from Sephadex G-25 Gel Filtration

Tube 3+4 plus	Protein per tube (mg)	Specific enzyme activity (ρKat×10^{-2}/mg.protein)
Control	0.063	0.8 ± 0.04
None	0.063	27.7 ± 2.06
Tube 1	0.063	27.9 ± 1.43
Tube 2	0.064	33.0 ± 1.43
Tube 5	0.064	22.2 ± 1.72
Tube 6	0.063	20.8 ± 0.80
Tube 7	0.063	18.6 ± 1.27
Tube 8	0.063	1.1 ± 0.16
Tube 9	0.063	1.0 ± 0.16
Tube 10	0.063	0.5 ± 0.16
Tube 11	0.063	0.5 ± 0.16
Tube 12	0.063	0.3 ± 0.06
Tube 13	0.063	0.7 ± 0.16
Tube 14	0.063	0.5 ± 0.05
Tube 15	0.063	0.3 ± 0.03
Tube 16	0.063	0.5 ± 0.16
Tube 17	0.063	0.2 ± 0.06
Tube 18	0.063	0.2 ± 0.03

Control was boiled 'None' which contained 0.25ml of the combination from tubes-3 and 4 plus 0.25ml of the preparation buffer;
The values for protein/tube are the means of three replicates and, the values for specific activity are the means of three replicates ± S.D., after correction for the scintillation blank and assay control.

It can be seen from Table 3.17 that the addition of each individual eluent to the combination of tubes-3+4 (named 'None') resulted in a different response with respect to enzyme activity over the control. When 0.25ml from tube -5, 6 or 7 was added to the combination of tubes-3+4, enzyme activity decreased slightly. Whilst, an obvious decrease in enzyme activity was detected when a combination from tubes-3+4 was added to 0.25ml of eluent from tube-8 to tube-18 inclusively. However, an improvement to enzyme activity was found when the mixture from tubes-3+4 was added to 0.25ml from tube-2 in which no enzyme activity was

detected (see Fig.3.25B in 3.7.5.1). The addition of 0.25ml from tube-1 to the combination tubes-3+4 caused no change in enzyme activity.

The results obtained show that any molecules eluted from the Sephadex G-25 column later than tube-4 did not promote nicotine *N*-demethylase activity, but inhibit the enzyme. However, the contents of tube-2 which have no enzyme activity increased enzyme activity when mixed with tubes-3+4. In considering that the fractionation range of the Sephadex G-25 column is between 1,000MW and 5,000MW, it would appear that molecules smaller than 5,000MW are unlikely to be involved in this *N*-demethylation. However, it would seem that there might be a molecule(s) involved with a MW greater than the enzyme(s), although further investigation is required.

3.7.6. Summary

The following points arising from the results presented in this section are now summarised:

(1) In a 20 day culture cycle, cells maintained in the dark exhibit a maximum nicotine *N*-demethylase activity around day 6 when culture growth is in the early period of linear increase.
(2) In contrast, the maximum activity of nicotine *N*-demethylase in light-cultured cells in a 20 day culture cycle appeared around day 10, when the growth of the culture was in the late period of linear increase.
(3) Following subcellular fractionation, most of enzyme activity (0.5ρKat) in the four fractions was present in the intermediate pellet, whilst the maximal specific activity (1.5ρKat/mg.protein) appeared to be associated with the microsomal fraction. TEM

observation shows that some membrane vesicles and/or fragments present in intermediate and microsomal fractions may be associated with this activity.

(4) Further characterisation of the enzyme using a dialysed enzyme preparation showed that this enzyme(s) appears to be NADPH dependent, but is unlikely to be a transmethylation.

(5) Fractionation of the enzyme preparation on a Sephadex G-25 gel column showed that molecules of greater size than the enzyme rather than smaller than 5,000MW are likely to be involved in nicotine *N*-demethylation.

Chapter Four:

DISCUSSION

Alterations to the chemical structure of added substances by cultured plant cells can result in the formation of either novel or known compounds and offers great potential for the production of useful chemicals. Over the last decade, the biochemical potential of plant cell cultures to produce a range of natural metabolites, including pharmaceuticals, flavours, pigments and agrochemicals, has been explored particularly with respect to their biotechnological and commercial applications (Anderson et al, 1986, Fowler, 1987, Yeoman et al, 1990 and Suga & Hirata, 1990). However, there are still only a few examples which have been a commercial success (Fowler, 1987). This is partially due to our limited knowledge of the biochemical mechanisms of many biotransformation reactions, although some of these reactions have been demonstrated in the laboratory to be effective in the bioconversion of added compounds (Barz, 1977 and Yeoman et al, 1990).

The bioconversion of nicotine to nornicotine, a reaction of important theoretical and commercial significance, has been studied to some extent. Our understanding of this *N*-demethylation has been enhanced not only through the use of intact tobacco plants, but also by the use of cell cultures. Very recently, the enzyme(s) catalysing this bioconversion has been demonstrated in the microsomal fraction from plants of *Nicotiana otophora* (Chelvarajan, et al, 1993). However, details of this reaction mechanism and especially the enzyme(s) responsible for the *N*-demethylation have not so far been revealed in cell cultures of *Nicotiana tabacum*, although the nature of the bioconversion has been studied (Barz et al, 1978, Hobbs & Yeoman, 1991a and Manceau et al, 1989). To remedy this gap in our knowledge, a detailed investigation of the *N*-demethylation of added nicotine to nornicotine in tobacco cell suspension cultures was begun in this laboratory over three years ago. This was the first occasion that the enzyme(s) responsible for *N*-demethylation of nicotine in a cell-free homogenate from tobacco cell cultures had been characterised and the enzyme(s) was

subsequently designated as nicotine *N*-demethylase. In this Chapter, the results obtained from this study are discussed together with previously published work, paying particular emphasis to nicotine *N*-demethylase and its enzymatic mechanism.

This discussion is divided into six Sections. The first considers the fate of nicotine added to cultured cells, together with an examination of extraction efficiency of nicotine and nornicotine and the possible existence of bound forms of the alkaloids. The kinetics of *N*-demethylation using both non-radioactive and radioactive nicotine are discussed in Section Two. In the Third Section, an assessment is made of the assay system developed for nicotine *N*-demethylase. Subsequently, in Section Four, the characterisation of nicotine *N*-demethylase is evaluated, together with an examination of the subcellular location of the enzyme and the effect of light on the activity of nicotine *N*-demethylase during a culture cycle. In Section Five, particular attention is paid to the mechanism of nicotine *N*-demethylation. In the final Section, future work is considered which could lead to a better understanding of this *N*-demethylation.

4.1. The Fate of Nicotine Added to Cell Suspension Cultures of Tobacco

A knowledge of the fate of nicotine added to cell cultures is an essential prerequisite to studies on its metabolism. In particular, the availability of this alkaloid to the centres of conversion within the cells must be considered. There are numerous reports in the literature on the detection and quantitation of the tobacco alkaloids from various tissues by a variety of methods (see Saunders & Blume, 1981). There is, however, little evidence of the efficiency of the methods employed for these extractions. It could well be that nicotine added to cell cultures might be bound to components within the cells or in the medium and become unavailable. Also, the

alkaloids may not be released during the extraction process (Manceau, et al, 1989). Therefore, it was necessary to re-examine each of the most popular methods of extraction and then choose the most efficient one for the study of nicotine *N*-demethylation.

4.1.1. Extraction of added nicotine and nornicotine from cell suspension cultures

In this study, five published methods were evaluated for their efficiency in the extraction of nicotine and nornicotine (see 3.2.1 and Table 3.1). The results obtained show that only three of the five methods tested gave acceptable results when the two alkaloids were measured by HPLC. Whilst, the extracts obtained using the procedures of Saunders & Blume (1981) (Method 2) and Manceau et al (1989) (Method 3) made the quantitation by HPLC impossible. In case of Method 2, the problem may lie in the fact that an extraction solvent consisted of methanol and 1N HCl in a ratio of 2:3 (v/v) was used in this experiment by misunderstanding the description of the method of Saunders & Blume (1981), who used a solvent of 40% (v/v) methanol containing 0.1% (v/v) 1N HCl. The very low pH of these extracts would seriously interfere with quantitative analysis by HPLC. However, the method used by Manceau et al (1989), if reported correctly, would have produced samples more suitable for analysis by GC than by HPLC.

Alkaloids are highly basic substances and are normally extracted from plant materials using a weakly acidic alcoholic mixture (prepared with 1N HCl or 10% acetic acid) and then precipitated with alkali, usually concentrated ammonia (Harborne, 1984). Under acidic conditions, the alkaloids become water soluble due to protonation of the *N* atom of the pyrrolidine ring, whilst the subsequent alkalisation causes deprotonation of the *N* atom promoting the transfer of the alkaloids to an

organic solvent. This general approach has been used to extract tobacco alkaloids from air dried tobacco leaves (Saunders & Blume, 1981) and fresh tobacco tissue (Tiburcio & Galston, 1987), with a better than 95% recovery of added nicotine and nornicotine. Also, Hobbs (1989) used this procedure to clean up extracts from tobacco cell cultures to remove non-alkaloid compounds. However, a re-evaluation of his method in this study showed that the recovery of known amounts of alkaloids added to cell cultures was only about 66% (±14%) for nicotine and 30% (±19%) for nornicotine, although HPLC performance for both alkaloids was satisfactory. This low recovery of both alkaloids, in particular of nornicotine, may be due to this lengthy extraction procedure (Tiburcio & Galston, 1987). Also, with Hobbs' method (1989), some of the added nicotine and nornicotine was not effectively released from the cells when they were first homogenised in ammonia-chloroform and where the alkaloids are likely to be associated with cell components due to their relative insolubility in water. Similar recovery rates (58% for nicotine and 27% for nornicotine) were obtained using the method of Horwitz (1970). This is a very simple extraction procedure in which frozen dried cells are ground up in ammonia and then extracted with chloroform. It would appear that deprotonation of the alkaloids by the addition of ammonia directly to cell cultures may not release all the added alkaloids due to the complexity of cell suspension cultures.

In order to explore whether the constituents of the culture medium or metabolites released from the cultures may affect the extraction of alkaloids, three 'media', i.e., distilled water, fresh B5 medium and the medium from 10 day old cultures, were compared using added nicotine and nornicotine (see Table 3.2). The results showed no marked differences between the recovery rates of nicotine and nornicotine for these three 'media', although the recovery of the two alkaloids did vary from replicate to replicate. This indicates that the constituents in the medium do not seriously affect

the extraction of the alkaloids. Recovery from extracts of fresh medium, however, is slightly better than from 10 day old medium, suggesting that some of the cellular metabolites present in the 10 day old medium may have some slight effect on alkaloid extraction.

A slight modification of the method of Lockwood & Essa (1984) gave the highest recovery rates of the five methods tested, about 81% for nicotine and 70% for nornicotine. HPLC performance was the same as that of Hobbs (1989), but the recovery variations were much smaller. This improvement in the recovery rate may be linked to the use of a strong alkali, such as potassium hydroxide (2N), which would cause deprotonation of the *N* atom of the alkaloids aiding distribution towards the organic phase. Also, some of the contaminating substances present in the extracts may be hydrolysed or removed by the alkali. In fact, 2N sodium hydroxide has been employed in the extraction of tobacco alkaloids from tobacco callus and leaves (Tiburcio & Galston, 1987). It is also possible that raising the temperature may improve the recovery of added alkaloids, since nicotine and nornicotine become more soluble in water at temperatures higher than 60°C (Budavari, 1989). This was presumably part of the reasoning behind the use of hot methanol by Barz and his co-workers (1978) to extract nicotine and nornicotine from cell cultures.

It is unfortunate that none of the five methods tested gives an extract recovery close to 100%. Of the methods tested, however, that of Lockwood & Essa (1984) was the best and therefore selected for the determination of nicotine and nornicotine in this study.

4.1.2. The possible presence of bound forms of nicotine in cell cultures

It has been demonstrated that bound forms of alkaloids are present in extracts of plants of *Conium* and *Papaver* (Waller & Nowacki, 1978 and Fairbairn & Steele, 1980). Also, bound forms of nicotine were presumed to be present in extracts of tobacco plants (Tso & Jeffery, 1961) and in tobacco cell cultures (Manceau, et al, 1989). In Manceau's work, evidence for the existence of bound forms of nicotine depended on the observation that the amount of added nicotine which disappeared from cell cultures was greater than the amount of nornicotine produced. With tobacco cell cultures, however, Hobbs & Yeoman (1991a) showed that close to 100% of the added nicotine could be accounted for by the nornicotine produced, a result which did not favour the presence of bound forms of nicotine. In contrast to their results, the lower recovery (81% for nicotine and 70% for nornicotine) of added alkaloids to cell suspension cultures seen in this study would suggest that some bound forms of nicotine may exist (see 4.1.1).

Experiments, in which ^{14}C-nicotine was added to 10 day old cell cultures for 24hr, showed that 92% of the added radioactivity could be accounted for with 75% in the chloroform phase. This is consistent with the results obtained on the recovery of added non-radioactive nicotine (see Table 3.1). Only 2.7% of the added radioactivity remained in the cell debris, suggesting that there may be slight binding of ^{14}C-nicotine to some of the cell components. This is in sharp contrast to the results of Manceau et al (1989). The 8% of added radioactivity unaccounted for in the study reported in this thesis is probably due to absorption onto the glassware during extraction.

The retention of 15% of the added radioactivity in the aqueous phase after extraction may not necessarily suggest the presence of nicotine conjugates, since nicotine and nornicotine added to distilled water could not be completely recovered into

chloroform (see Table 3.2). It would appear that this retention is not related to bound forms of the alkaloids or extensive binding to cellular components as presumed by Manceau et al (1989). In addition, filtration through Sephadex G-25 gel showed only one peak on the elution profile (see 3.4.1 and Fig.3.9) with no evidence of free and bound forms of nicotine. It was also shown that mild hydrolysis with 1M NH_4Cl followed by vigorous hydrolysis with 2M HCl (Morris, et al, 1991) did not effectively improve the recovery of ^{14}C-nicotine, providing further evidence that the conjugates do not exist in these cell cultures.

From these findings, it is concluded that extensive binding of nicotine is unlikely to occur in tobacco cell cultures. Therefore, the distribution of nicotine between the aqueous and organic phase during extraction with an organic solvent appears to depend on the physico-chemical properties of the molecule.

4.2. Kinetics of *N*-Demethylation of Added Nicotine to Nornicotine

The kinetic pattern of *N*-demethylation of added nicotine to nornicotine in tobacco cell cultures of *N. tabacum* (Hobbs & Yeoman, 1991a) and *N. plumbaginifolia* (Manceau, et al, 1989) has been studied in some detail. In order to confirm these results, first of all, the optimal nicotine feeding point in a culture cycle was determined. Then, after re-examination of the kinetic pattern, a further approach was adopted in which ^{14}C-nicotine was employed to reveal the early-state kinetics of this *N*-demethylation.

4.2.1. Determination of the optimal nicotine feeding point in the culture cycle

The evidence presented in 3.2.2 shows that the addition of 10mg of nicotine at different points in a culture cycle followed by in a 24hr incubation leads to the production of different amounts of nornicotine. The maximal bioconversion activity was found between day 5 and day 10 (Fig.3.3b), although the maximum amount of nornicotine produced per culture (flask) appeared at day 10 (Fig.3.3a). As there is a limited amount of biomass available at day 5, it was decided to compromise between the amount of biomass and bioconversion capacity, day 10 of the culture cycle was therefore selected as the optimal nicotine feeding point.

In the literature, there are reports that nicotine has been added at different points in the culture cycle of tobacco cell suspension cultures. For example, day 0 (Manceau, et al, 1989 and Hobbs, 1989), day 7 (Barz, et al, 1978) and day 10 (Hobbs & Yeoman, 1991a). However, there is no comment in these reports on why these feeding points were selected. Ideally, the optimal nicotine feeding point in a culture cycle should coincide with the maximum bioconversion activity of added nicotine to nornicotine as well as the availability of the biomass.

It is also important that the amount of nicotine added is not toxic to the cultures. Indeed, it was shown that nicotine up to 160mg per 50ml cultures was not harmful to the cells (see Fig.3.4). An even higher nicotine tolerance by cell cultures of *N. plumbaginifolia* was reported by Manceau et al (1989), in which the amount of nicotine used was 10g/l fresh cells (corresponding to 500mg per a 50ml culture).

The results obtained suggest that this 10 day old cell culture fulfils the requirements of being a successful biotransformation system as stated by Steck & Constabel (1974) and Yeoman et al (1990). That is: 1) The culture must have the enzymes necessary for the transformation of precursor to product; 2) The product must be

formed faster than it is further metabolised; 3) The culture must tolerate the added precursor and the product.

4.2.2. Kinetics of nicotine bioconversion to nornicotine

In this study, 70% of the added nicotine (62.5μmol) had been converted to nornicotine after 15 days incubation (see Fig.3.5). This is consistent with the results of Hobbs & Yeoman (1991a), who demonstrated, using this cell line, a 66% conversion of 62.5μmol of added nicotine after 14 days. Hobbs & Yeoman (1991a) also showed that, in a comparison of this mixotrophic cell line with others, i.e. a heterotrophic and a photoheterotrophic line, a better than 90% conversion was achieved. They claimed that the difference in the amount of biomass could account for the decreased level of bioconversion in the mixotrophic culture. Indeed, the evidence presented here (see 3.2.6) shows clearly that increasing the biomass from 6g to 9g (fresh weight) per flask improved the conversion of added nicotine to 100%. Also, a 100% conversion was observed when 5mg of nicotine was added to 6g fresh weight of cells. These results agree with the suggestion of Hobbs & Yeoman (1991a) that the bioconversion capacity of the 10 day old mixotrophic cultures (about 6g in fresh weight) is exceeded by 10mg (62.5μmol) of nicotine. Thus, as has been shown, either an increase in biomass or a decrease in the amount of nicotine raises the bioconversion capacity of the cultures to 100%.

From the detailed breakdown of the amount of nicotine and nornicotine present in cells and medium (see Fig.3.6a,b), it can be seen that during the first 5hr, the nicotine content of cells increases slightly paralleling a decrease in the medium. This indicates that the added nicotine has been taken up by the cells. Simultaneously with the decrease in nicotine content in both cells and medium, there is an increase in

nornicotine which increases gradually until a maximum value is reached at the end of 15 days incubation, when a detectable amount of nicotine is still present in the cultures. This would account for the unconverted amount of added nicotine, correlating with the maximal conversion of 70% observed in this culture. These results are consistent with those reported by Hobbs & Yeoman (1991a), suggesting that nicotine bioconversion appears to be intracellular, with nicotine being taken up from the medium, and converted to nornicotine which is subsequently released into the medium.

However, it was also observed that, during the first day after the addition of 10mg nicotine to the cultures, about 4mg of the added nicotine has disappeared while, less than 1mg of nornicotine has appeared. Such a discrepancy was previously observed in the study of Manceau, et al (1989), who explained it by invoking nicotine binding to cell components (only a 53% conversion of added nicotine by cell cultures of *N. plumbaginifolia*). In this study with *N. tabacum*, however, there is no evidence of extensive nicotine binding, suggesting that experimental error resulting from a low extraction efficiency of nicotine and nornicotine may have been the cause of this discrepancy. Indeed in a later paper, Manceau, et al, (1992) reported a 100% conversion of added nicotine using an improved method of analysis and reinterpreted the 53% conversion as poor analytical performance. In this study, there is no suggestion that the nornicotine formed was degraded to any extent, which conflicts with the report of Manceau et al (1992). However, some further metabolism of the nornicotine formed is possible and this is discussed next together with the results of experiments in which ^{14}C-nicotine was used to study this bioconversion.

4.2.3. Kinetics of ^{14}C-nicotine bioconversion

It is possible that added nicotine may be transformed into compounds other than nornicotine and that nornicotine may be metabolised. To establish the early-state kinetics of this bioconversion, 0.5μCi of ^{14}C-nicotine was incubated for 72hr with a 10 day old cell culture. From these results (see Fig.3.12), it was shown that bioconversion take places within 1hr when more than 40% of the added ^{14}C-nicotine was taken up by the cells. Simultaneously, a small amount of ^{14}C-nornicotine appeared in the cultures. These results are similar to those reported by Barz and his co-workers (Barz, et al, 1978), in which about 50% of the added ^{14}C-nicotine was taken up by all four cell lines tested within 30 to 75min. Thereafter, the ^{14}C-nornicotine increased sharply paralleling the decrease in ^{14}C-nicotine in the cultures until an equilibrium was reached at around 6hr. However, the balance between the disappearance of nicotine and appearance of nornicotine was not as good as that reported by Hobbs and Yeoman (1991a), because small amounts of several compounds other than nornicotine were labelled, although ^{14}C-nornicotine was the predominate labelled product. (The identification of these labelled compounds is examined in a later section of this discussion.) It was also observed that most of the ^{14}C-nornicotine produced appeared in the cell fraction rather than in the medium at all sampling times. This is consistent with the observations of Barz and his co-workers (1978), but inconsistent with the observation of Hobbs & Yeoman (1991a) who showed that 93% of the alkaloids were released into the medium. This difference could be related to the fact that the added ^{14}C-nicotine (9.5×10^{-3}μmol) was diluted out considerably by the endogenous nicotine, since the concentration of added ^{14}C-nicotine was only approximately 1/160 of the endogenous nicotine at that stage of the culture cycle. This 'diluting effect' could also explain why only around 70% of added ^{14}C-nicotine was converted into nornicotine (see Fig.3.11) by the end of the incubation, as this difference could not be explained by a low percentage bioconversion.

Therefore, it can be concluded that the results obtained in this study would support the suggestion presented in 4.2.2 and that of Hobbs and Yeoman (1991a) that the bioconversion of nicotine is intracellular.

4.3. The Occurrence of Nicotine *N*-Demethylase and the Development of an *in vitro* Enzyme Assay

From the evidence available in the literature, it would appear that the bioconversion of nicotine to nornicotine in either plants or cell cultures of tobacco is catalysed enzymatically (Leete & Chedekel, 1974; Poulton, 1981 and Wallex & Nowacki, 1978). To elucidate details of this enzymatic reaction, it was first necessary to demonstrate the existence of the enzyme(s) responsible for this bioconversion. Recently, the enzyme(s) has been reported in the microsomal fraction from leaves of *N. otophora* (Chelvarajan, et al, 1993). In this study, the presence of this enzyme(s) in cell-free extracts prepared from cultured tobacco cells was reported by Hao & Yeoman (1993). In view of the suggestion that the term demethylation can be used to describe two general reactions: transmethylation and demethylation (Poulton, 1981), this enzyme(s) was tentatively designated as nicotine *N*-demethylase. However, further proof of the enzyme(s) and its characteristics depended on the development of a reliable *in vitro* enzyme assay which is now discussed.

4.3.1. General considerations in the development of an assay

The first step in the development of an enzyme assay system is to detect the enzyme activity in a cell-free preparation. This involves identification of the source of the enzyme and selection of an adequate buffer system (Wilson, 1986). In view of the

observation by Hobbs and Yeoman (1991a) that 10 day old cell cultures are capable of converting added nicotine to nornicotine with high efficiency, it was decided to use 10 day old cultures as a source of the enzyme nicotine *N*-demethylase.

In preliminary trials, no activity was detected in cell-free extracts prepared with 0.1M potassium phosphate buffer (pH7.5), and supplemented with either a variety of additives such as sucrose, DTT, EDTA, ascorbate, glycine, magnesium sulphate and manganous sulphate or their combinations. However, activity was detected at pH9.0 when a 0.1M Tris-HCl buffer, supplemented with 0.25M sucrose, 3mM DTT and 5mM EDTA, was used. Activity was also obtained using 0.1M Tris-HCl buffer (pH6.0-9.0) and 0.1M Glycine-NaOH buffer (pH8.5-10.0). Using these buffers, it was shown that the pH optimum of this reaction lies between pH9.0 and 9.5. This unusual pH optimum is further discussed in the section on characterisation of the enzyme. It was also shown that buffers and reagents prepared with double-distilled water were more effective in the improvement of enzyme activity than those prepared with distilled water. This is probably due to the fact that some contaminants present in single-distilled water may inhibit the enzyme during cell disruption (Cooper, 1977a).

It can also be seen for all enzyme assays that there was a small amount of radioactivity corresponding to nornicotine in the boiled controls. This is due in part to an impurity in the ^{14}C-nicotine (97.7%, supplied by *NEN*, Du Pont) and tailing of the spots separated by TLC (see Fig.2.3). This problem was also observed by Chelvarajan et al (1993), in their studies on nicotine *N*-demethylase with isolated microsomes of tobacco leaf tissue, which they claimed was a result of non-enzymatic demethylation.

4.3.2. Protection of the enzyme(s) during cell disruption

It is well established that oxidation of sulfhydryl groups on the enzyme protein and the presence of undesirable metal ions during preparation of cell-free extracts can be countered by the addition of thiol compounds and EDTA respectively (Cooper, 1977a and Wilson, 1986). These were confirmed in this study (see 3.5.1) where it was shown that 3mM DTT and 5mM EDTA protect cell extracts from these effects. However, an excess amount of these reagents results in denaturation of the enzyme.

Other undesirable effects, such as oxidation by quinone compounds, polarity of the buffer medium or degradation of the enzyme by proteases, must also be taken into consideration. It is well known that these deleterious effects on enzyme activity can be reduced or prevented by the addition of specific compounds. The fact that 0.25M sucrose has no effect on enzyme activity whilst 1% DMSO is inhibitory, suggests that the enzyme requires a mild hydrophobic environment, as a decrease in polarity and ionic strength of the buffer medium by the addition of 0.25M sucrose did not affect the activity of the enzyme. However, excessive depolarisation caused by the addition of DMSO partially inactivated the enzyme. Oxidation caused by the presence of quinone compounds could not be prevented by the addition of 5% PVP, since it was found to inactivate the enzyme. However, PVPP (0.2g/g.tissue), a cross-linked form of PVP, was successfully employed by Chelvarajan et al (1993) in studies on this enzyme with isolated microsomes from tobacco leaves. These differences which are difficult to explain may be due to the different source of the enzyme and the different preparation buffers used. Also in this study, PMSF was found to seriously denature the enzyme, although it can be used to inhibit protease activity (Cooper, 1977a).

Apart from DTT and EDTA, none of the other chemicals at the concentrations used in this study protect the enzyme during cell disruption, although while 0.25M of sucrose has no effect on enzyme activity it may be useful when density differential centrifugation or frozen enzyme preparations are employed.

4.3.3. Solubilisation of the enzyme(s)

One of the approaches used to solubilise the enzyme(s) was to add detergents or salt during the preparation of homogenates. Accordingly, the enzyme extracts were prepared in the presence of Triton X100 (0.1%), CHAPS (0.3%), deoxycholate (0.1%), and NaCl (0.5M). However, virtually no demethylation of the added nicotine was detected in the presence of these reagents. A similar negative effect of CHAPS, a zwitterionic detergent, was also observed by Chelvarajan, et al (1993), although 0.3% of CHAPS has been reported to dissociate with high efficiency a *N*-methyltransferase involved in vindoline biosynthesis from the thylakoid membranes of chloroplasts (Dethier & De Luca, 1993). Also, the addition of these chemicals to the preparation buffer did not improve the yield of protein with the exception of Triton X100. However, the presence of this detergent made chloroform extracts so sticky that the alkaloids could not be separated effectively on TLC and also seriously affected the protein assays. In contrast, Triton X100 did give satisfactory results with nicotine *N*-demethylase in tobacco leaves (Chelvarajan, et al, 1993). Consequently, no chemical additives other than EDTA and DTT were employed in the routine assay.

One interesting observation was that variation of the pH in the extraction buffer significantly increased protein solubility. A maximum value was obtained in the alkaline buffer. This coincided with the maximum enzyme activity obtained at

pH9.0. It is likely that raising the pH of the extraction buffer increased the yield of protein, because pH alters the binding of protein to the cell wall and membranes releasing associated proteins into the soluble phase.

Therefore, it was possible to solubilise the enzyme by centrifugation at the appropriate pH, as the enzyme appears to be loosely associated with the cell wall or membranes. Indeed, the amount of soluble protein in the extract was raised significantly as the time of centrifugation was increased up to 10min, whilst the maximum enzyme activity was observed when the enzyme extract was centrifuged at 8,000*g* for 1min. However, increasing the time of centrifugation tended to reduce enzyme activity. This may be due in part to an increase in the amount of some of the competitive proteins or inhibitors. Alternatively, some small fragments of cell wall or membrane to which the enzyme is bound may be further precipitated by prolonged centrifugation, leading to a decreased availability of enzyme in the supernatant. Since differential centrifugation separates particles not only according to size but also on the basis of density, an increase in the time of centrifugation will cause smaller particles to pellet (Rickwood, 1984). In addition, machine temperature produced by prolonged centrifugation might cause some denaturation of the enzyme, since a conventional Eppendorf centrifuge was used in a cold room at 4°C. Consequently, centrifugation of the enzyme preparation at 8,000*g* for 1min resulted in two fractions, supernatant and pellet, with most of the enzyme activity present in the supernatant rather than in the pellet (see Fig.3.11).

From these results, it may be concluded that an enzyme assay procedure using ^{14}C-nicotine is satisfactory. Using this procedure, the enzyme activity in the supernatant was 9.1 times the control and superior to a crude extract. The further characterisation of the enzyme(s) is now examined in the next section.

4.4. Characterisation of Nicotine *N*-Demethylase

4.4.1. Studies on the kinetics of nicotine *N*-demethylation

(1) *pH optimum* The pH optimum for this enzyme lies between 9.0 and 9.5. This rather high pH optimum is considered unusual for a plant enzyme, although an alkaline range was previously observed for putrescine *N*-methyltransferase (pH8-9) by Mizusaki et al (1971). However, a lower pH optimum of 7.0-7.5 was reported for a similar enzyme in isolated microsomes from tobacco leaf tissue by Chelvarajan et al (1993) and, also, for aminopyrine *N*-demethylase (7.5) by Fonne-Pfister et al (1988) and for *p*-chloro-*N*-methylaniline *N*-demethylase in avocado pear (7.6-8.0) by Dohn & Krieger (1984). Such a high pH optimum observed in this study for nicotine *N*-demethylase from tobacco cell cultures is at present unclear. It would appear that the pH optimum of *N*-demethylation may depend on the specificity and source of the enzyme, despite the fact that the enzyme(s) appears to catalyse the same reaction. Whilst, this high pH is probably accounted for by the endogenous level of alkaloids present in the cells.

(2) *Temperature optimum* The temperature optimum for *N*-demethylation lies between 25°C and 30°C. An increase in temperature to 37°C reduced enzyme activity by 70%. This is also the optimum temperature for nicotine *N*-demethylase in isolated microsomes from tobacco leaf tissue reported by Chelvarajan and his co-workers (1993) and lies within a range common for a wide range of plant enzymes because of the physiological significance of the temperature (Waller & Dermer, 1981 and Wilson, 1986).

(3) *Relationship between protein concentration and enzyme activity* To ensure the compilation of an accurate set of kinetic data for this reaction under a given set of conditions, it was necessary to determine the range of protein concentration over which enzyme activity was proportional. This was established between 0.11-0.22mg of protein per reaction tube. Below 0.11mg per tube, enzyme activity was not proportional. This may be due to the presence of the inhibitor(s), since a crude enzyme extract was employed. In addition, it might be accounted for by an error arising from the protein assay, as each assay was routinely carried out by measuring 20µl of the enzyme solution and then converting the value to 0.5ml (see 2.7.4), the lower the concentration, the less accurate the determination. In fact, enzyme assays were performed routinely at a protein concentration between 0.2-0.4mg.protein/ml enzyme preparation except where indicated otherwise.

(4) *Time course of nicotine N-demethylation* It has been established that the rate of nicotine *N*-demethylation increases linearly with time up to 30min. Thereafter, the reaction rate gradually becomes constant until the end of the 90min incubation. Although a non-linear phase for up to 60min was observed for *p*-chloro-*N*-methylaniline *N*-demethylase in avocado pear (Dohn & Krieger, 1984), a similar linear pattern was reported for nicotine *N*-demethylase in a microsomal fraction from tobacco leaves (Chelvarajan et al, 1993).

However, the results obtained in this study are not completely consistent with those of Chelvarajan et al (1993), where the reaction rate decreased rapidly after 30min and was almost zero after 4hr incubation. It was also unclear whether further degradation of the nornicotine formed occurred in the study of Chelvarajan et al (1993), which could complicate interpretation of their results. It is well known that the reaction rate of a typical enzymatic reaction increases with time and subsequently remains

constant (Wilson, 1986), unless degradation of the product occurs. In fact, the degradation of nornicotine formed from added nicotine is negligible (Barz, et al, 1978; Manceau, et al, 1989; Hobbs & Yeoman, 1991a and this study).

(5) *Kinetics of nicotine N-demethylation* The kinetic properties of nicotine *N*-demethylase were studied when the enzyme was assayed at 30°C varying the amount of nicotine from 0 to 15.2μM per tube at pH9.0 for 30min. The resulting Michaelis-Menten kinetic plot is presented in Fig.3.20 and shows a clear relationship between the concentration of substrate and the reaction velocity. The regression of the reciprocals of the two sets of kinetic data resulted in a linear plot with a 99.2% coefficient of determination, suggesting that the kinetic data fulfil the criteria of a Lineweaver-Burk plot (see Fig.3.21). From these data, the K_m and V_{max} were estimated to be 7.4μM and 7.6×10^{-2}ρKat respectively. However, an analysis of the errors involved in the collection of the data and hence in the determination of the parameters K_m and V_{max} shows that there is a highly non-uniform distribution of error over the range of values of $1/V$ and 1/[S] in the Lineweaver-Burk plot (Cornish-Bowden, 1979). To minimise the non-uniformity, the use of other plots, in particular the Hanes plot, has been recommended (Cornish-Bowden, 1979). For this reason, a Hanes plot was produced in this study using the same sets of the kinetic data used for the Lineweaver-Burk plot. However, the regression coefficiency of these data to fulfil a linear plot was only 97.5%. Therefore, the kinetic parameters estimated from the Lineweaver-Burk plot with a higher regression coefficiency (99.2%) were adopted.

In addition, it can be seen that a higher K_m (51μM) and V_{max} (24ρ mol/min.mg.protein) were observed for nicotine *N*-demethylase in the isolated microsomes from tobacco leaf tissue (Chelvarajan, et al, 1993). This may be because

a higher concentration of enzyme of 2mg/ml was used in their study than 0.2mg/ml in this study. Also, the assay was performed in the presence of NADH and NADPH, a coenzyme which was found to stimulate enzyme activity (this will be discussed in the next section). However, the employment of the unit for V_{max} (ρ mol/min.mg.protein) in their studies is subject to criticism, because kinetic studies are usually performed within a linear range of enzyme concentration where reaction velocity is independent on enzyme concentration. Thus, the correct unit for V_{max} should always be either ρmol/min or ρmol/sec (ρKat).

4.4.2. Requirements of co-factors and co-enzymes

In preliminary experiments, it was shown that nicotine *N*-demethylation did not appear to require added ATP, β-NAD and β-NADP at the concentrations tested, but did require β-NADPH. Indeed, a reasonably high level of enzyme activity was detected in the crude enzyme preparations without any of these additives. In such a crude extract, it is likely that there are a variety of molecules aiding enzyme activity. When a dialysed enzyme preparation was used, the results largely confirmed the preliminary results with the crude homogenate, indicating that nicotine *N*-demethylation not only requires β-NADPH but probably also β-NADH. However, the removal of the endogenous co-factors or co-enzymes by dialysis from the enzyme preparations caused a sharp decrease in enzyme activity and this activity could only be recovered by the addition of β-NADPH and β-NADH, with the former much more effective than the latter. Unexpectedly, Sephadex G-25 gel filtration of the enzyme preparation failed to separate out such small participating molecules (see Table 3.17). However, this does not rule out a requirement for β-NADPH and β-NADH, because these co-enzymes may be bound to the polypeptide chain of the enzyme as a

prosthetic group (Wilson, 1986) which may be removed by dialysis but not by gel filtration.

This requirement for the reduced form of pyridine nucleotide has also been observed with other *N*-demethylases from a variety of plant sources, including ρ-chloro-*N*-methylaniline *N*-demethylase (Dohn & Krieger, 1984 and O'Keefe & Leto, 1989), aminopyrine *N*-demethylase (Fonne-Pfister, et al, 1988), chlorotoluron *N*-demethylase (Mougin, et al, 1990) and nicotine *N*-demethylase (Chelvarajan, et al, 1993), suggesting that nicotine *N*-demethylation in tobacco cultured cells may involved cytochrome P-450. This point will be discussed later in this chapter.

4.4.3. Subcellular fractionation of nicotine *N*-demethylase

Fractionation of the crude enzyme homogenate by differential centrifugation shows that the highest nicotine *N*-demethylase activity is present in the intermediate pellet. However, the highest specific enzyme activity per mg.protein of all four fractions was in the microsomal pellet which contained only a small amount of protein. A parallel investigation using TEM revealed obvious differences between the microsomal and intermediate fractions, which would suggest that nicotine *N*-demethylase occurs in two intracellular structures common to both fractions, such as the membrane vesicles and/or fragments (see Fig.3.24). The small amount of enzyme activity in the cell wall fraction may be accounted for by the presence of a few number of vesicles or unbroken fragments with enzyme activity, due to incomplete maceration in the pestle and mortar.

However, the results presented here are to some extent different from most of the published observations in which *N*-demethylase is usually present in the microsomal

fraction prepared by centrifugation of post-mitochondrial supernatants at 100,000*g* (Dohn & Krieger, 1984, Fonne-Pfister, et al, 1988, O'Keefe & Leto, 1989, Mougin, et al, 1990 and Chelvarajan, et al, 1993). This subcellular location is one characteristic of the involvement of cytochrome P-450 in *N*-demethylation (West, 1980 and Donaldson & Luster, 1991). The causes of the observed discrepancy may be due to: (1) cross contamination which is very common with differential centrifugation (Griffiths, 1986); (2) inactivation or degradation of the enzyme during the fractionation process and (3) experimental errors arising from both enzyme and protein assay. Also, the difference in the source of the plant tissue used may result in a different fractionation pattern (Simpkins, 1986). From this uncertainty, therefore, it is clear that more precise techniques such as density gradient centrifugation and immuno-histochemistry are required to reveal the true subcellular nature of nicotine *N*-demethylase.

4.4.4. Effect of light on nicotine *N*-demethylation

The results presented show that light caused a 26% increase in the maximum enzyme activity in comparison with dark-cultured cells. This result is similar to that reported by Manceau et al (1989), but is opposite to the observation of Hobbs & Yeoman (1991a) who reported an inhibitory effect of light on the bioconversion. The lower *N*-demethylation in cells grown in the dark in Manceau's study could apparently be due to analytical errors reported in their later work (Manceau, et al, 1992), rather than the explanation given by Hobbs & Yeoman (1991a) that cells grown in the light were actively growing and the biotransformation occurs to a greater degree later in the culture cycle, when the cells start to senesce. Hence, cells grown in the light should have a shorter and more rapid growth phase than cells grown in the dark (Hobbs, 1989). In fact, the growth pattern of the mixotrophic cell line used in this

investigation was similar in both light and dark and, the maximum enzyme activity in light-cultured cells occurred at day 10 when culture growth was still in the linear phase.

However, there is not sufficient evidence to conclude that this *N*-demethylation is bound to a photo-dependent system as reported by Manceau et al (1989). Indeed, the promotion in nicotine *N*-demethylase activity seen in this study is probably due to the fact that the photosynthetic system in mixotrophic cultures is activated when grown in the light, resulting in an increase in oxygen content which is simultaneously taken up by the cells to support the *N*-demethylation system. Since, it is well known that nicotine *N*-demethylation occurs in parallel with the uptake of oxygen (Waller & Nowacki, 1978 and Poulton, 1981). In comparison with dark-cultured cells, the delay in the point at which maximum enzyme activity is reached in light-cultured cells may also account for the involvement of oxygen as speculated, because oxygen content increases in parallel with photosynthesis as a result of the increase in biomass when the cultured cells were approaching the end of the linear phase of growth. This may coincide with the 'indirect effect' of light on nicotine *N*-demethylation as presumed by Hobbs & Yeoman (1991a). If this is the case, it might be suggested that nicotine *N*-demethylation in tobacco cell cultures is involved with the cytochrome P-450 system, since a molecular oxygen requirement is one of the criteria for cytochrome P-450 involvement (West, 1980; Donaldson & Luster, 1991).

4.5. Mechanism of Nicotine *N*-Demethylation

Attention will now be paid to the possible mechanism of nicotine *N*-demethylation, in which the methyl group on the pyrrolidine ring of the nicotine molecule

disappears. Three aspects of evidence will examined separately before a conclusion is reached.

4.5.1. Transmethylation?

If a transmethylation is involved, there must be an effective methyl group acceptor as nicotine is a methyl group donor in nicotine *N*-demethylation. However, the addition of some selected methyl group acceptors at a range of concentrations such as glycine, ethanolamine and putrescine, to crude homogenates of tobacco cell cultures showed no positive effect on enzyme activity and, indeed, ethanolamine was found to inactivate the enzyme. In contrast to this result, James (1975) reported that bioconversion took place in a tobacco leaf homogenate in the presence of glycine and ethanolamine, suggesting that this reaction might be a transmethylation. In addition, it has been shown that putrescine is a methyl group acceptor in the biosynthesis of the pyrrolidine moiety catalysed by putrescine *N*-methyltransferase (Mizusaki, et al, 1971). However, putrescine did not improve nicotine bioconversion in cell-free extracts in this study. This may be because putrescine *N*-methyltransferase is an *S*-adenosyl-*L*-methionine-dependent enzyme which normally shows a surprisingly strict specificity toward only one class of substrate or to a limited number of closely related compounds, and to a specific position in the substrate molecule (Luckner, 1990a).

Further studies using dialysed enzyme preparations showed that these methyl group acceptors had no positive effect on nicotine *N*-demethylase activity. Also, the results obtained from Sephadex gel filtration experiments (see 3.7.5.2) confirm that, apart from the exceptions already discussed, molecules smaller than 5,000MW are unlikely to be involved in *N*-demethylation. It is therefore concluded that the evidence

obtained does not favour a *N*-transmethylation, despite the fact that some reports in the early years supported the view that this process was a transmethylation (Leete & Bell, 1959) or a partial transmethylation (Kiasaki & Tamaki, 1961b and Blaim & Ciszewska, 1973).

4.5.2. Stereochemical evidence

One of the important hypotheses concerning the mechanism of nicotine *N*-demethylation was made by Leete and Chedekel in 1974, in which they presumed that this reaction involved the opening and subsequent closing of the pyrrolidine ring during the conversion (see Fig.1.2). This hypothesis was based on the observation of Kisaki & Tamaki (1961a) that the nornicotine derived from optically pure (-)-nicotine in tobacco plants was partially racemized. In tobacco cell cultures, however, the evidence obtained from analysis by polarimetry and chiral GC show convincingly that nornicotine produced from added nicotine is only one enantiomer exclusively (see Table 3.4 and Fig 3.8), suggesting that nicotine *N*-demethylation by tobacco cell cultures does not involve ring opening. Three points may be put forward to support this view:

(1) *The reliability of Leete and Chedekel's hypothesis* There has been no strong evidence to support the proposed mechanism of nicotine *N*-demethylation since Leete and Chedekel made their hypothesis in 1974. According to this hypothesis, a stable intermediate, i.e., (-)-nicotine-*N'*-oxide, is involved in nicotine *N*-demethylation. Although this is a common metabolite of (-)-nicotine detected in animals (Booth & Boyland, 1971; Nwosu, et al, 1988), this presumed intermediate of the bioconversion in plants is currently unknown. Also, a study of the metabolism of nicotine-*N'*-oxide in excised *N. glutinosa* leaves indicated that nornicotine was not

produced directly from nicotine-*N'*-oxide, but formed by a reduction through nicotine (Alworth, et al, 1969). In support of this assumption, Leete (1977) reported that the conversion of labelled nicotine-*N'*-oxide to nornicotine in *N. glutinosa* and *N. glauca* plant was very small (1.4%) compared with the incorporation of nicotine to nornicotine (49%). Accordingly, an alternative mechanism involving no opening of the pyrrolidine ring was presumed by Leete (1977), where the racemic nornicotine was converted from (-)-nicotine through a tautomeric shift, an iminium salt (see Fig.1.3) and this tautomerism is possible *in vitro*. From these findings, it would appear that the ring-opening hypothesis is no longer tenable.

(2) *The mechanism is different between the tobacco plant and its cultures* According to the evidence obtained in this study, it would seem that the bioconversion of added (-)-nicotine to (-)-nornicotine in plant cell cultures does not conform to the hypothesis of Leete and Chedekel (1974). In view of the suggestion made by Yeoman (1987) that the overall level of metabolic control in plant cell cultures may be different from that in the plant, it is possible that the physiological and biochemical characteristics of cell cultures may differ from the intact plant. Therefore, it might be predicted that there are two different mechanisms, one in the plant and another in plant cell cultures. Although the possibility of the existence of two mechanisms has already been suggested by Stepka & Dewey (1961), there is no evidence to support this speculation.

(3) *An alternative mechanism* In principle, opening of the pyrrolidine ring during the conversion can only yield complete racemized nornicotine [50% of (+) form and 50% of (-) form], unless an asymmetric molecule is involved in some way in the mechanism which could result in more of one enantiomer than the other. This asymmetric molecule is likely to be an enzyme as already presumed by Leete and

Chedekel (1974). Coincidentally, the enzyme(s) catalysing nicotine *N*-demethylation has recently been demonstrated in the tobacco plant (Chelvarajan, 1993) and in tobacco cell cultures in this laboratory (Hao & Yeoman, 1993). This aspect already discussed in the previous section will be developed in later sections.

Tracer studies revealed the presence of small amounts of metabolites other than nornicotine formed from added ^{14}C-nicotine. One of these was tentatively identified by GC-MS as *N'*-formylnornicotine, whilst the identities of the other two remain unknown. *N'*-Formylnornicotine has been isolated from Burley tobacco (Warfield ,et al, 1972) and also found in tobacco cell cultures at the end of a 30 days incubation with nicotine (Hobbs & Yeoman, 1991a). There is a possibility that it is an intermediate of nicotine *N*-demethylation. It has been established that oxidative elimination of the *N*-methyl group of a substrate initiated by hydroxylation to form a hydroxymethyl derivative may be spontaneously decomposed into product and formaldehyde (Luckner, 1990b). The resulting hydroxymethyl derivative may also undergo further oxidation leading to *N'*-formylnornicotine (Leete, 1977). Alternatively, the resulting formaldehyde may react with the nornicotine formed to produce *N'*-formylnornicotine. However, this could occur spontaneously, since the reaction between a -NH group and formaldehyde occurs readily in organic synthesis. Thus, the rate for the production of *N'*-formylnornicotine should be much lower than enzymatic nicotine *N*-demethylation, which had been observed in this study (see Fig.3.10), conclusively, eliminating the possibility of *N'*-formylnornicotine as an intermediate in nicotine *N*-demethylation.

4.5.3. Enzymological evidence

The occurrence of nicotine *N*-demethylase in tobacco cell cultures (Hao & Yeoman, 1993) and in isolated microsomes of tobacco leaves (Chelvarajan, et al, 1993) demonstrated for the first time that the bioconversion of nicotine to nornicotine is an enzymatically oxidative *N*-demethylation. Some of the evidence presented in this study is consistent with the involvement of cytochrome P-450 in oxidative *N*-demethylation of nicotine (see 4.4). This possibility is now examined.

As reviewed in the Introduction (see 1.5.3), cytochrome P-450 is a multi-form protein complex which mediates a wide range of reactions in plants (West, 1980 and Donaldson & Luster, 1991). The involvement of cytochrome P-450 in nicotine *N*-demethylation has been suggested from studies on isolated microsomes from tobacco leaves by Chelvarajan et al (1993). Also, some other *N*-demethylations have been shown to be linked with cytochrome P-450, such as ρ-chloro-*N*-methylaniline *N*-demethylase (Dohn & Krieger, 1984 and O'Keefe & Leto, 1989), aminopyrine *N*-demethylase (Fonne-Pfister, et al, 1988) and chlorotoluron *N*-demethylase (Mougin, et al, 1990).

In tobacco cell cultures, the evidence obtained shows that this reaction requires β-NADPH and β-NADH, although β-NADH was less effective than β-NADPH. The requirement of molecular oxygen for nicotine *N*-demethylation was implicated from the results of the 'indirect effect of light' (see 4.4.4). These results provide some tentative indirect evidence for the involvement of cytochrome P-450 (see 1.5.3) and further investigations are necessary. For instance, β-NADPH kinetic studies would be required with a constant concentration of nicotine and the establishment of reaction stoichiometry, which is, 1mole each of β-NADPH, oxygen and nicotine consumed for 1mole of nornicotine produced (West, 1980).

In addition, it has been observed in this study that the subcellular location of nicotine *N*-demethylase in tobacco cell cultures is distinctive from those demethylases found in plants which are usually present in microsomal preparations after centrifugation of the post-mitochondrial supernatants at 100,000*g* (Dohn & Krieger, 1984; O'Keefe & Leto, 1989; Fonne-Pfister, et al, 1988; Mougin, et al, 1990 and Chelvarajan, et al, 1993). However, cytochrome P-450 is an elusive group of enzymes in plants and the subcellular location of plant cytochrome P-450 is diverse (West, 1980). Although the microsomal fraction has been employed as the starting material in many experiments, the composition of a microsomal fraction would depend on the homogenisation techniques used and the abundance of the different types of membranes in the source tissue (Donaldson & Luster, 1991). In fact, the subcellular origins of the microsomal membranes have not been demonstrated to any extent in tobacco cell cultures. Therefore, the evidence obtained in this study, although very tentative, does not rule out the involvement of cytochrome P-450 in nicotine *N*-demethylation.

The results obtained in this study indicate that some of the characteristics of this *N*-demethylation are consistent with cytochrome P-450 involvement. However, the mechanistic features of the plant cytochrome P-450-dependent monoxygenases have not been characterised nearly as thoroughly as the hepatic microsomal system (West, 1980). The present findings warrant further investigation.

4.5.4. Conclusions

According to the results presented in this study and the corresponding discussions, it can be concluded that the bioconversion of added nicotine to nornicotine by tobacco cell cultures is likely to be an oxidative demethylation catalysed by nicotine *N*-demethylase. This oxidative enzyme(s) appears to be a monooxygenase, or

sometimes referred to as a mixed-function oxidase (Young & Beevers, 1976 and Butt & Lamb, 1981), which may be associated with cytochrome P-450. This oxidative *N*-demethylation of nicotine to nornicotine may be initiated by the hydroxylation of the *N*-methyl group of the nicotine molecule. During this process, cytochrome P-450 is reduced with cytosolic β-NADPH by NADPH:cytochrome P-450 reductase (another number of cytochrome P-450 complex) donating two electrons to a molecule of oxygen and, subsequently, one oxygen atom hydroxylates with the *N*-methyl group and the other forms water. The resulting hydroxy derivative is unstable and is non-enzymatically decomposed to nornicotine and formaldehyde. Accordingly, a proposed mechanism of this reaction is illustrated in Fig.4.1.

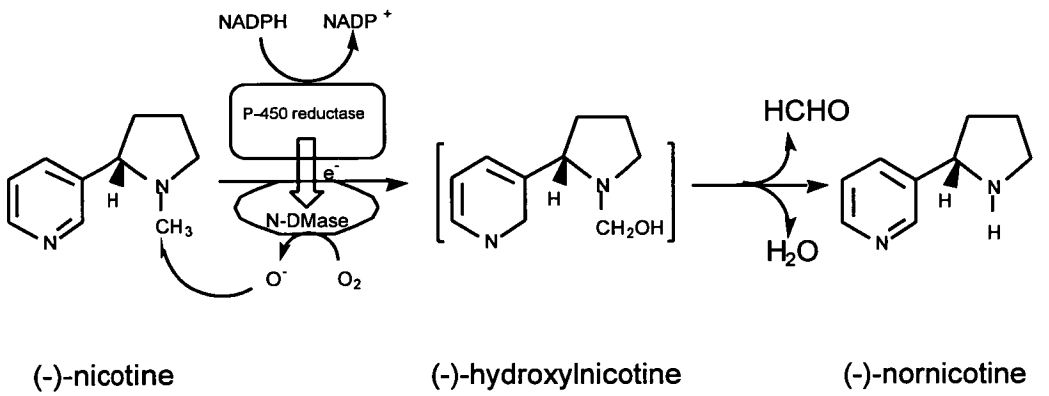


Fig.4.1. A Proposed Mechanism of Nicotine *N*-Demethylation
N-DMase: nicotine *N*-demethylase

Although many of the findings to date are consistent with this reaction in either the tobacco plant or its cultured cells, there still remain many uncertainties. For example, whether cytochrome P-450 is definitely involved in this reaction; how the uncompleted racemization of nornicotine is accomplished; how the enzymatic reaction is regulated in the cell and so on. Further investigations on the mechanism of

this bioconversion are therefore necessary to answer these uncertainties and some suggestions are made in the next section.

4.6. Future work

The work presented in this thesis has begun to forge an understanding of nicotine *N*-demethylation in tobacco cell cultures. For future work, investigations using radioisotopic techniques, enzyme techniques and immunochemical techniques should be addressed to further examine established information, aiming at elucidating a realistic mechanism of this reaction. This could be achieved by addressing the objectives outlined below:

4.6.1. Radiochemical approach

To determine the fate of the methyl group of nicotine, methyl carbon labelled or $^{14}C/^{3}H$ double labelled nicotine should be employed in feeding experiments. With the methyl carbon labelled, it should be possible to show whether the radioactivity is located exclusively in the formaldehyde formed, as presumed by the oxidative mechanism. This could be trapped as its semicarbazole and measured by scintillation counting (Polland & Nebert, 1973). With $^{14}C/^{3}H$ double labelled nicotine, not only the fate of the methyl carbon could be traced, but also the fate of the methyl hydrogen would be revealed. This should establish whether the metabolism of the methyl hydrogen fulfils the proposed oxidative mechanism (see Fig.4.1) through a determination of radioactive distribution in the reaction system.

4.6.3. Further characterisation of nicotine *N*-demethylase

In this part of the future work, attention should mainly be paid to ascertain whether cytochrome P-450 is involved in nicotine *N*-demethylation in tobacco cell cultures. Accordingly, the following questions must be addressed:

(1) Is there a CO-binding pigment in the reduced enzyme preparation with a maximal absorbance at 450nm in the CO-difference spectrum?
(2) Is nicotine *N*-demethylation promoted in the presence of molecular oxygen?
(3) Can the promotion by oxygen in (2) be inhibited when CO takes the place of oxygen in the reaction?
(4) Can the inhibition by CO be reversed by light with a maximum in the photoaction spectrum at 450nm?
(5) Establish the stoichiometry of nicotine *N*-demethylation, is it 1mole each of β-NADPH, oxygen and nicotine utilised for each mole of nornicotine formed? Here, the purified enzyme must be used to avoid possible competing reactions.
(6) Is one oxygen atom from molecular oxygen (e.g., using $^{18}O_2$) incorporated into one mole of nornicotine?
(7) Is nicotine *N*-demethylation activity affected by alterations to the activities of other cytochrome P-450-linked enzymes, such as NADPH:cytochrome P-450 reductase, NADH:cytochrome b^5 and its reductase?

It has already been mentioned in the Introduction (see 1.5.3) that not all of these criteria need to be satisfied in any one system to prove cytochrome P-450 involvement, but it is necessary to meet several of them in order to make a convincing case (West, 1980)

4.6.3. Subcellular localisation of nicotine *N*-demethylase

To further determine the subcellular localisation of nicotine *N*-demethylase, density gradient centrifugation should be adopted. It is known that gradient centrifugation is better than the differential centrifugation used in this study to reduce possible cross contamination. For precise determination, the availability of antibodies against nicotine (Langone, et al, 1973) and nornicotine (Fliniaux, et al, 1992) should help to discover the reaction binding site. Whilst, immunohistochemical localisation using antibodies raised against nicotine *N*-demethylase, or specific cytochrome P-450 if it is involved, would be especially useful for a definite identification of subcellular location. Such a study would depend on the establishment of purified nicotine *N*-demethylase and/or the specific cytochrome P-450.

4.6.4. Purification of nicotine *N*-demethylase

The purification of nicotine *N*-demethylase from tobacco cell cultures is vital to many aspects of further investigations leading to the elucidation of the reaction mechanism. Having established in this study that the maximum activity was present at day 10 of the culture cycle when the cells were maintained in the light, the 10 day old cells are a satisfactory starting source for the purification. This is an empirical work which usually relies on information from subcellular location, the fractionation pattern and the characteristics of the enzyme. If this enzyme(s) is Cytochrome P-450-linked protein(s), however, its isolation and characterisation can be difficult due to the nature of Cytochrome P-450 (Donaldson & Luster, 1991)

4.6.5. Long term prospects

In the long term, if the involvement of cytochrome P-450 is established, nicotine *N*-demethylase and the corresponding cytochrome P-450 proteins should be isolated and purified from tobacco cell cultures. It would be expected that the production of antibodies against these proteins should soon lead to the identification of the genes which encode this enzyme(s). The subsequent genetic manipulation of these genes could be useful tools for engineering tobacco cell cultures and tobacco plants to satisfy human requirements.

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PUBLICATION

XV International Botanical Congress

Yokohama, Japan

PACIFICO YOKOHAMA

August 28-September 3, 1993

ABSTRACTS

第十五回国際植物科学会議組織委員会

The 15th International Botanical Congress 1993, Yokohama (Japan)
Poster Section: No. 4037

BIOCONVERSION OF ADDED (-)-NICOTINE TO (-)-NORNICOTINE BY CELL SUSPENSION CULTURES OF *Nicotiana tabacum* L cv. Wisconsin-38.

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The bioconversion of nicotine to nornicotine, a natural phenomenon of important commercial significance, has been investigated in tobacco cell cultures. The properties of (-)-nornicotine produced from (-)-nicotine by 10 day old cell cultures have been determined using both, polarimetry and chiral gas chromatograghy. The results obtained, which are convincingly consistent, show that the nornicotine produced is exclusively one enantiomer. This provides strong evidence that the bioconversion of nicotine by tobacco cultures does not involve opening of the pyrrolidine ring. It is suspected that the mechanism of nicotine bioconversion by cultured cells might differ from that in the plant in which a partially racemized mixture of (+)- and (-)-nornicotine was detected, this could be the result of the opening and closing of the pyrrolidine ring during bioconversion.

Radioactive feeding experiments, in which *DL*-[pyrrolidine-*2'*-^{14}C]-nicotine was added to 10 day old cultures, showed that the bioconversion was intracellular and that nornicotine was the major product. The appearance of nornicotine paralleled the disappearance of the added nicotine, although small amounts of four other radioactive-metabolites were observed. The identification of the four metabolites using Mass Spectrometry is in process.

It is concluded from enzymological studies using cell free extracts of 10 day old cultured cells that the bioconversion of nicotine to nornicotine is enzyme-controlled and enantioselective. This is the first time that this enzyme has been reported and characterised. Further enzymological studies are now being carried out.